Investigating progestin-mediated crosstalk between the androgen receptor and estrogen receptor subtypes in breast cancer cell lines

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Declaration

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ABSTRACT

Estrogen and the estrogen receptor alpha (ERα) are traditionally considered as the main etiological factors in breast cancer. However, other members of the steroid receptor family, including ERβ and the androgen receptor (AR) have also been shown to play a role. It is known that ERα drives breast cancer cell proliferation, while ERβ antagonizes ERα-mediated effects. Moreover, the AR, which is expressed in the majority of ER-positive breast cancer tumours, has been shown to inhibit the transcriptional activity of ERα and increase ERβ expression when activated by the potent natural androgen, 5α-dihydrotestosterone (DHT). Together, this suggests that the AR is associated with a good prognosis in ER-positive breast cancer. The question that arises is whether all agonists binding to the AR would elicit similar effects. This is particularly relevant to progestins used by millions of women in contraception and menopausal hormone therapy (HT), as a number of progestins are known to bind to the AR, with some displaying androgenic properties similar to DHT and others displaying anti-androgenic properties. For example, progestins like medroxyprogesterone acetate (MPA), norethisterone acetate (NET-A), and levonorgestrel (LNG) have been shown to be as potent and efficacious as the natural androgen DHT, while others like nestorone (NES) and nomegesterol acetate (NOMAC) display anti-androgenic properties similar to the natural progestogen, progesterone (P_4). It is noteworthy that MPA, NET-A and LNG have all been associated with an increased risk of breast cancer. However, the underlying mechanisms whereby these progestins contribute to increased breast cancer risk has not been established. In this study, our main aim was to investigate whether androgenic progestins, unlike anti-androgenic progestins, would elicit similar effects as DHT and the synthetic androgen, mibolerone (Mib), on ERβ and ERα expression in human breast cancer cell lines. First however, we used mammalian two-hybrid assays to investigate the ability of the progestins to induce the ligand-dependent interaction between the NH_2- and COOH-terminal domains (N/C interaction) of the AR, and showed that progestins elicit different conformations in the receptor. Western blot analysis showed that unlike the androgens that increased AR protein levels, the progestins did not influence AR protein levels in the MCF-7 BUS or MDA-MB-453 breast cancer cells. Quantitative real-time PCR (qPCR) showed that like the androgens, MPA, NET-A and LNG all increased ERβ mRNA expression in the MDA-MB-453 cell line, while P_4, NES and NOMAC did not. Moreover, by using the AR antagonist, hydroxyflutamide, we showed that these effects were mediated by the AR. Although these results suggest another mechanism by which the AR may inhibit breast cancer cell growth, the results should be interpreted with caution as we show that the AR-mediated effects of the androgens and androgenic progestins, in fact, increased proliferation of the MCF-7 BUS and T47D breast cancer cell lines. Unlike, Mib and DHT, which decreased ERα mRNA expression via the AR, MPA, NET-A and LNG had no effect on ERα expression. Although the

precise physiological implications of these preliminary results remain to be determined, our findings highlight the fact that the role of progestins in breast cancer is not straightforward. Moreover, these findings contribute to our understanding of crosstalk between the AR and ER subtypes in breast cancer.

OPSOMMING

Estrogeen en die estrogeenreseptor alfa (ERα) word tradisioneel beskou as die hoof etiologiese faktore in borskanker. Ander lede van die steroïedreseptor familie, insluitende ERβ en die androgeen reseptor (AR), speel egter ook 'n rol in borskanker. Dit is bekend dat ERα borskankerproliferasie dryf, terwyl ERβ hierdie ERα-gemedieërde effekte teenwerk. Verder is dit bewys dat die AR, wat in die meerderheid van ER-positiewe borskankergewasse uitgedruk word, die transkripsionele aktiwiteit van ERα inhibeer en die uitdrukking van ERβ verhoog wanneer dit geaktiveer word deur die natuurlike androgeen, 5α-dihidrotestosteroon (DHT). Saam dui dit daarop dat die AR geassosieer word met 'n goeie prognose in ER-positiewe borskanker. Die vraag wat egter ontstaan is of alle agoniste wat aan die AR bind soortgelyke effekte sal ontlok. Hierdie vraag is veral van toepassing op progestiene wat deur miljoene vroue gebruik word in voorbehoedmiddels en menopausale hormoonterapie (HT), aangesien baie van hierdie progestiene aan die AR kan bind, en óf androgeen eienskappe soortgelyk aan DHT vertoon, óf anti-androgeniese eienskappe. Byvoorbeeld, progestiene soos medroksieprogesteroon asetaat (MPA), noretisteroon asetaat (NET-A) en levonorgestrel (LNG) is bewys om ewe sterk en doeltreffend as die natuurlike androgeen DHT te wees, terwyl ander soos nesteroon (NES) en nomegesterolasetaat (NOMAC) anti-androgeniese eienskappe soortgelyk aan die natuurlike progestogeen progesteroon (P_4) vertoon. Dit is opmerklik dat MPA, NET-A en LNG almal verband hou met 'n verhoogde risiko van borskanker. Die onderliggende meganismes waardeur hierdie progestiene bydra tot 'n verhoogde borskanker risiko is egter nog nie vasgestel nie. In hierdie studie was ons hoofdoel om vas te stel of androgeniese progestiene, in teenstelling met anti-androgeniese progestiene, soortgelyke effekte as DHT en die sintetiese androgeen, miboleroon (Mib), op ERβ- en ERα-uitdrukking in menslike borskanker sellyne sal hê. Eerstens het ons die vermoë van die progestiene ondersoek om die ligand-afhanklike interaksie tussen die NH_2- en COOH-terminale domeine (N/C interaksie) van die AR te induseer. Ons het gewys dat progestiene verskillende konformasies in die reseptor ontlok. Deur gebruik te maak van westernklad-analise het ons gewys dat progestiene, in teenstelling met die androgene wat AR-proteïenvlakke verhoog het, geen invloed op AR-proteïenvlakke in die MCF-7 BUS of MDA-MB-453 borskankerselle gehad het nie. Kwantitatiewe intydse PKR (qPKR) het getoon dat MPA, NET-A en LNG, nes die androgene, ERβ mRNA-uitdrukking in die MDA-MB-453-sellyn verhoog, terwyl P_4, NES en NOMAC geen effek getoon het nie. Verder, deur die gebruik van die AR antagonis, hidroksieflutamied, het ons getoon dat hierdie effekte deur die AR bemiddel is. Alhoewel hierdie resultate 'n ander meganisme voorstel waarmee die AR borskankergroei kan inhibeer, moet dié resultate met omsigtigheid geïnterpreteer word, aangesien ons toon dat die AR-gemedieërde effekte van die androgene en androgeniese progestiene inderdaad die proliferasie van die MCF-7 en T47D-sellyne verhoog. In teenstelling met

Mib en DHT, wat via die AR die ERα mRNA uitdrukking verlaag, het MPA, NET-A en LNG geen effek op ERα-uitdrukking gehad nie. Alhoewel die presiese fisiologiese implikasies van hierdie voorlopige resultate nog bepaal moet word, beklemtoon ons bevindings die feit dat die rol van progestiene in borskanker nie eenvoudig is nie. Daarbenewens dra hierdie bevindinge by tot ons begrip van wisselwerking tussen die AR en ER subtipes in borskanker.

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ALPHABETICAL LIST OF ABBREVIATIONS

AF-1	activation function-1
AF-2	activation function-2
AF-3	activation function-3
AI	aromatase inhibitor
ANOVA	analysis of variance
AP-1	activator protein-1
AR	androgen receptor
ARE	androgen response element
ATCC	American Type Culture Collection
Bical	bicalutamide
bp	base pair
CEE	conjugated equine estrogen
CFP	cyano fluorescent protein
ChIP	chromatin immunoprecipitation
C_q	quantification cycle
CS-FCS	charcoal-stripped fetal calf serum
DBD	DNA-binding domain
DEPC	diethyl pyrocarbonate
Dex	dexamethasone
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide
DRSP	drospirenone
E. coli	Escherichia coli
E_1	estrone
E_2	17β-estradiol
E_2V	E_2-valerate
E_3	estriol
EDTA	ethylenediaminetetra-acetic acid
EE	ethinyl estradiol
EMSA	electrophoretic mobility shift assay
ERE	estrogen response element

ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
EtOH	ethanol
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GES	gestodene
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HDL-C	high-density lipoprotein cholesterol
HIV-1	human immunodeficiency virus
HRP	horseradish peroxidase
HSV-2	herpes simplex virus type 2
HT	hormone therapy
ICI	fulvestrant (ICI 182, 780)
LB	Luria-Bertani medium
LBD	ligand-binding domain
LDL-C	low-density lipoprotein cholesterol
LNG	levonorgestrel
MAPK	mitogen-activated protein kinase
Mib	mibolerone
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
MRE	mineralocorticoid response element
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NES	nesterone
NET-A	norethisterone acetate
NET-EN	norethisterone enanthate
NFκB	nuclear factor-kappa B
NOMAC	nomegestrol acetate
NTD	N-terminal domain
OD	optical density
OHF	hydroxyflutamide
P_4	progesterone
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate-buffered saline
PCOS	polycystic ovary syndrome
PI3K	phosphatidylinositol 3-kinase
PR	progesterone receptor
PRE	progesterone response element
qPCR	quantitative real-time polymerase chain reaction
R1881	methyltrienolone
R5020	promegestone
RLU	relative light unit
RO	reverse osmosis
rpm	revolutions per minute
RU486	mifepristone
SARM	selective AR modulator
SDS	sodium dodecyl sulfate
SERD	selective ER downregulator
SERM	selective ER modulator
SRE	steroid response element
T	testosterone
TAT	tyrosine aminotransferase
TBS	tris-buffered saline
TBST	tris buffered saline tween
TNBC	triple negative breast cancer
VEGF	vascular endothelial growth factor
VTE	venous thromboembolism
YFP	yellow fluorescent protein

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Chapter 1

Literature Review

1.1   Introduction

Progestins are synthetic compounds designed to mimic the biological actions of the natural progestogen, progesterone (P_4), via the progesterone receptor (PR). Derived from different parent compounds, progestins were designed in four consecutive generations (Schindler *et al*. 2003; Sitruk-Ware 2006; Africander *et al*. 2011a; Stanczyk *et al*. 2013). The first generation progestins include medroxyprogesterone acetate (MPA) and norethisterone enanthate (NET-EN) or norethisterone acetate (NET-A), while levonorgestrel (LNG) forms part of the second generation. Gestodene (GES), a derivative of LNG, is classified as a third generation progestin, while the fourth and newest generation include drospirenone (DRSP), nestorone (NES) and nomegesterol acetate (NOMAC).

Progestins have a wide range of therapeutic applications, the most common being its use in contraception and hormone therapy (HT) (Sitruk-Ware, 2006). In contraception, progestins suppress ovulation and inhibit sperm penetration in order to prevent pregnancy (Sitruk-Ware, 2006), while progestins used in HT prevent estrogen-induced endometrial hyperplasia in menopausal women with an intact uterus (Sitruk-Ware, 2006). Other therapeutic applications of progestins include the treatment of gynecological disorders like polycystic ovary syndrome (PCOS) (Archer and Chang, 2004; Guido *et al.*, 2004; Ehrmann, 2005) and endometriosis (Harrison and Barry-Kinsella, 2000; Irahara *et al.*, 2001; Vercellini *et al.*, 2003). Despite their therapeutic benefits, the use of some progestins are associated with side-effects such as an increased risk for cardiovascular disease, venous thromboembolism (VTE), increased susceptibility to genital tract infections, and increased risk of invasive breast cancer (Mostad *et al.*, 2000; Rossouw *et al.*, 2002; Beral *et al.*, 2003; Fournier *et al.*, 2008; Morrison *et al.*, 2010).

Many of these side-effects are suggested to be due to the cross-reactivity of progestins with steroid receptors other than the PR (Hapgood *et al*. 2004; Sitruk-Ware 2006; Africander *et al*. 2011a; Stanczyk *et al*. 2013; Louw-du Toit *et al*. 2017a). For example, it has been shown that MPA and GES exhibit glucocorticoid agonist activity, while P_4, NET-A, LNG and DRSP do not (Africander *et al*. 2011b; Stanczyk *et al*. 2013; Koubovec *et al*. 2005). Furthermore, DRSP (Krattenmacher, 2000), unlike MPA and NET-A (Africander *et al*. 2011b, and references therein), has been shown to elicit anti-mineralocorticoid activity, while NET-A, LNG and GES have all been shown to bind to estrogen receptor (ER)-α, but not ERβ (Louw-du Toit *et al*. 2017a). MPA, NET-A, LNG, GES, NES, NOMAC and DRSP have all been shown to bind the androgen receptor (AR) (Africander *et al*. 2014; Louw-du Toit *et al*. 2017a), which is the most widely expressed steroid receptor in breast cancer (Søreide *et al.*, 1992; Garay and Park, 2012). However, while MPA, NET-A, LNG and GES display androgenic properties similar to the natural androgen 5α-dihydrotestosterone (DHT), NES, NOMAC

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and DRSP display anti-androgenic properties similar to P_4 (Africander *et al*. 2014; Louw-du Toit *et al*. 2017a).

Multiple steroid receptors have been implicated in breast cancer biology, and these receptors often influence each other's activity (reviewed in (Sikora 2016)). For example, the DHT-activated AR has been shown to inhibit ERα transcriptional activity, resulting in the inhibition of ER-positive breast cancer cell growth (Peters *et al.*, 2009). In addition, the androgen-activated AR also inhibited breast cancer cell growth by increasing ERβ expression (Rizza *et al.*, 2014). Considering that progestins like MPA, NET-A and LNG display similar androgenic properties as the natural androgen DHT (Africander *et al*. 2014; Louw-du Toit *et al*. 2017a), the question arises whether these progestins would also increase ERβ expression. Considering the wide-spread expression of the AR in breast cancer tumours and its association with a favorable prognosis and lower tumour grade in ER-positive breast cancer (Castellano *et al.*, 2010; Hu *et al.*, 2011; Park *et al.*, 2011; Garay and Park, 2012; Tsang *et al.*, 2014), therapies targeting AR signaling are actively being researched (reviewed in (Rahim & O'Regan 2017)). Thus, if progestins displaying androgenic activity elicit similar effects as the androgens in breast cancer, these synthetic compounds may in fact be beneficial rather than harmful in breast cancer. Indeed, MPA has previously been used to treat breast cancer (Blossey *et al.*, 1984; Etienne *et al.*, 1992; Yamashita *et al.*, 1996), and the AR was required for these effects (Birrell *et al*. 1995a). It is therefore crucial to elucidate the mechanism of action of progestins binding to the AR, in order to understand how these ligands may either contribute to the development and progression of breast cancer or possibly be used in breast cancer treatment.

1.2   Progestins

It is well-known that the natural progestogen, P_4, has a relatively short biological half-life due to its rapid metabolism *in vivo* (Speroff and Darney, 1996). Synthetic progestogens, known as progestins, were thus designed to mimic the biological actions of P_4 via the PR, and have a longer half-life and higher bioavailability than P_4 (Sitruk-Ware, 2006; Stanczyk *et al.*, 2013). Progestins were designed in four consecutive generations, with the fourth generation progestins designed to be more selective in terms of binding to the PR (Stanczyk 2003; Schindler *et al*. 2003; Sitruk-Ware 2006; Africander *et al*. 2011a; Stanczyk *et al*. 2013). Numerous progestins with distinctly different structures are clinically available. Examples of the first generation progestins include MPA, and NET-EN or NET-A. NET-EN is a derivative of NET used in contraception, while NET-A is the acetate ester of NET used in both oral contraception and menopausal HT (Schindler *et al.*, 2003; Stanczyk, 2003; Sitruk-Ware and Plu-Bureau, 2004). Both NET-EN and NET-A are prodrugs that are metabolically converted to the biologically active NET (Stanczyk and Roy, 1990; Garza-Flores *et al.*, 1991; Kuhnz

et al., 1997). LNG, a derivative of NET, forms part of the second generation progestins, while GES, a derivative of LNG, forms part of the third generation progestins (Sitruk-Ware, 2006). The fourth and newest generation include progestins like NES, NOMAC and DRSP (Schindler *et al.*, 2003; Stanczyk, 2003; Stanczyk *et al.*, 2013). Progestins are derived from different parent compounds like P_4 and testosterone (T) (Figure 1.1). Those that are structurally related to P_4 include either 17α-hydroxyprogesterone derivatives like MPA, or 19-norprogesterone derivatives like NES and NOMAC. Progestins that are structurally related to T include 19-nortestosterone derivatives like NET-EN/NET-A, LNG and GES (Schindler *et al.*, 2003; Stanczyk, 2003; Stanczyk *et al.*, 2013). DRSP is the only progestin that is derived from the MR antagonist, spironolactone (Krattenmacher, 2000).

 Many progestins elicit their biological activities via binding to not only the PR, but also other steroid receptors such as the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), androgen receptor (AR), and estrogen receptor alpha (ERα) (Koubovec *et al*. 2005; Sitruk-Ware 2006; Stanczyk *et al*. 2013; Africander *et al*. 2013; Africander *et al*. 2014; Louw-du Toit *et al*. 2017a). Moreover, these progestins often elicit differential effects when binding to these receptors. For example, MPA and GES, unlike NET-A and LNG, have been shown to display glucocorticoid-like activity (Africander *et al*. 2011b; Stanczyk *et al*. 2013; Koubovec *et al*. 2005). In addition, DRSP displays potent anti-mineralocorticoid activity, while MPA and NET-A do not (Krattenmacher 2000; Africander *et al*. 2011a; Africander *et al*. 2013). MPA, NET-A, LNG and GES can also display androgenic properties, while NES, NOMAC and DRSP display anti-androgenic properties (Africander *et al*. 2014; Louw-du Toit *et al*. 2017a). Lastly, NET-A, LNG and GES, but not MPA, NES, NOMAC and DRSP, have been reported to display partial estrogenic activity by binding to estrogen receptor ERα, but not ERβ (Louw-du Toit *et al*. 2017a).
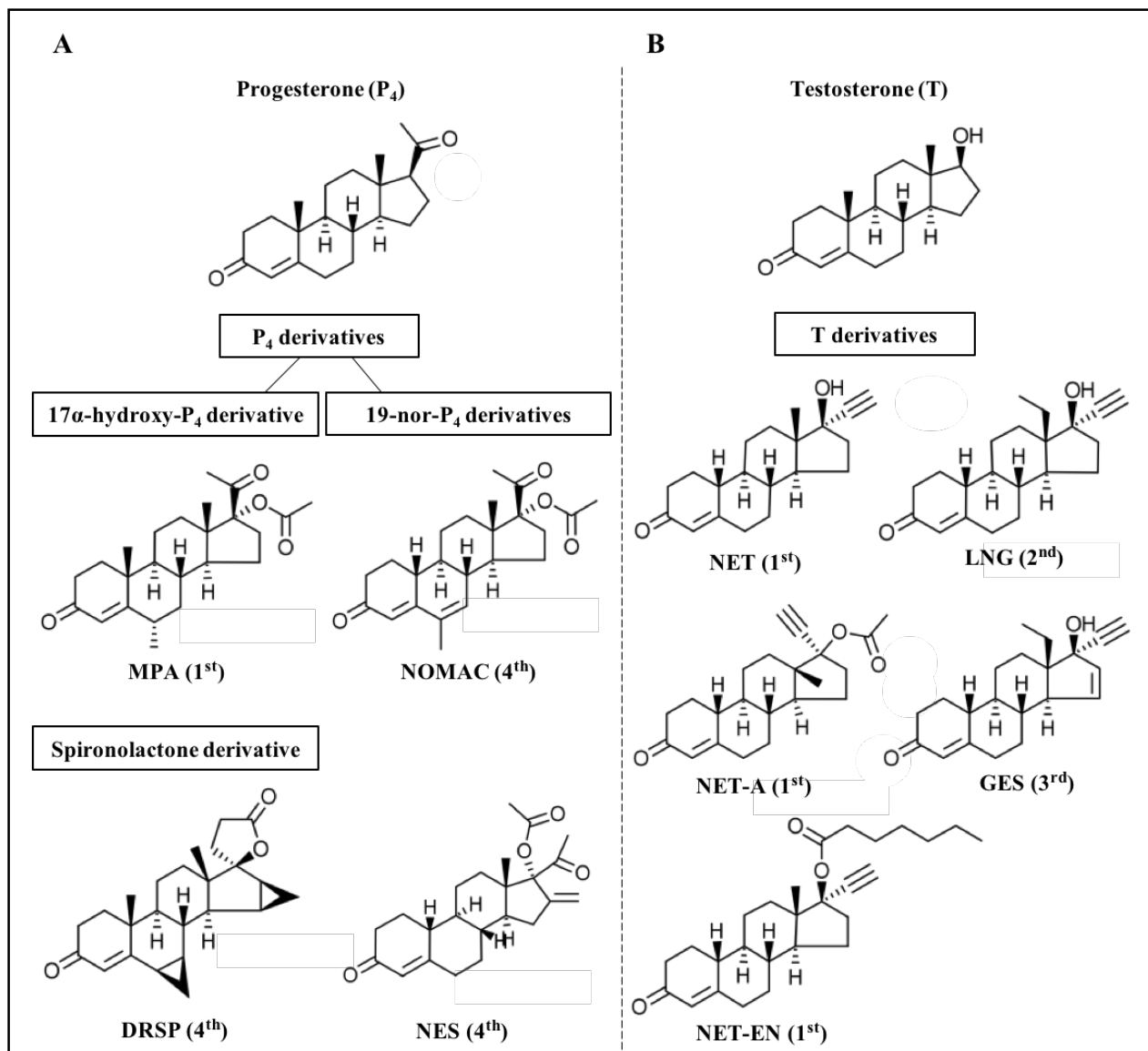
Figure 1.1. Structures of selected progestins from four consecutive generations. Progestins structurally related to (A) progesterone (P$_4$) include either 17α-hydroxyprogesterone derivatives like the 1st generation progestin medroxyprogesterone acetate (MPA), or 19-norprogesterone derivatives like the 4th generation progestins nestorone (NES) and nomegesterol acetate (NOMAC). The spironolactone derived progestin, drospirenone (DRSP), also forms part of the 4th generation progestins. Progestins structurally related to (B) testosterone (T) include the 1st generation progestins norethisterone enanthate (NET-EN)/norethisterone acetate (NET-A), 2nd generation progestins like levonorgestrel (LNG), and 3rd generation progestins like Gestodene (GES) are also structurally related to T. The structures used in this figure are from (Louw-du Toit *et al*. 2017b).

1.2.1 Therapeutic applications

Progestins have a wide range of therapeutic applications, such as the treatment of PCOS (Archer and Chang, 2004; Guido *et al*., 2004; Ehrmann, 2005), endometriosis (Harrison and Barry-Kinsella, 2000; Irahara *et al*., 2001; Vercellini *et al*., 2003) and menstrual disorders such as dysfunctional and

irregular uterine bleeding, menorrhagia (heavy menstruation) and dysmenorrhea (painful menstruation) (Williams and Creighton, 2012). In addition, some progestins are used to treat skin disorders such as acne vulgaris and hirsutism (Rosen *et al.*, 2003; Archer and Chang, 2004; Guido *et al.*, 2004; Batukan and Muderris, 2006; Krunic *et al.*, 2008; Olutunmbi *et al.*, 2008). Interestingly, at high doses of 500 – 1500 mg/day, some progestins like MPA have been used in cancer therapy (Blossey *et al.*, 1984; Etienne *et al.*, 1992; Yamashita *et al.*, 1996). However, these compounds are most commonly used in contraception and menopausal HT (Kuhl, 2005), which will be discussed in the following sections.

1.2.1.1 Contraception

Progestins prevent pregnancy by inhibiting ovulation, causing endometrial atrophy and altering the cervical mucus to prevent sperm penetration and fertilization (Greydanus *et al.*, 2001; Sitruk-Ware, 2006; Sitruk-Ware and Nath, 2010). These compounds are either administered in combination with an estrogen, such as E_2 or ethinyl estradiol (EE), or used as progestin-only contraceptive agents (Solter, 1999; Greydanus *et al.*, 2001; Erkkola and Landgren, 2005). Contraceptives are available in various doses and can be administered via different routes. For example, it can either be administered orally, via intramuscular injections, transdermal gels, patches or sprays, through implants or intrauterine devices (Toppozada *et al.*, 1983; Brache *et al.*, 2000; Greydanus *et al.*, 2001; Sitruk-Ware, 2006; Sitruk-Ware and Nath, 2010; Sitruk-Ware *et al.*, 2013).

The first generation progestins, MPA and NET-EN, are the most commonly used injectable contraceptives amongst females in South Africa (Draper *et al.*, 2006). While MPA, also referred to as Depo-Provera®, is administered intramuscularly at a dose of 150 mg every three months, NET-EN (Noristerat®) is administered at a 200 mg dose every two months (Toppozada *et al.*, 1983; Draper *et al.*, 2006). NET-EN is hydrolyzed to NET and other metabolites, which together have contraceptive action (Stanczyk and Roy, 1990; Garza-Flores *et al.*, 1991; Kuhnz *et al.*, 1997). In contrast, MPA itself is the active contraceptive agent (Mishell, 1996). The second generation progestin, LNG, is frequently encountered in the Mirena® intrauterine system, which delivers 12–20 µg LNG per day (Backman *et al.*, 2004; Fraser, 2013), but can also be used as an emergency oral contraceptive within 24 hours before, or up to 120 hours after, unprotected sexual intercourse (Cheng *et al.*, 2008). LNG is also available in a vaginal gel that can be applied before sexual intercourse to induce ovary dysfunction (Brache *et al.*, 2007; Massai *et al.*, 2007). Other progestins like the third generation progestin, GES, is commonly used in transdermal patches containing 1.9 mg GES in combination with 0.9 mg EE (Heger-Mahn *et al.*, 2004). However, it has also been used in combined oral contraceptive pills such as Minulet®, which contains 0.075 mg GES in combination with 0.03 mg

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EE (Kirkman *et al.*, 1994). Although not active as an oral contraceptive (Schindler *et al.*, 2003), NES is the most potent anti-ovulatory compound amongst the progestins when parenterally administered (Kumar *et al.*, 2000; Nath and Sitruk-Ware, 2009). NES can be administered either alone (75–100 μg/day) or in combination with EE (150 μg NES + 15 μg EE) in a sustained-release vaginal ring (Sitruk-Ware *et al.*, 2003). The fourth generation progestin, NOMAC is administered either in combination with E_2 in oral contraceptive agents such as Zoely® (2.5 mg NOMAC + 1.5 mg E_2) (Burke, 2013), or alone in subdermal implants (Uniplant®) (Coutinho *et al.*, 1996; Devoto *et al.*, 1997). DRSP, is commonly used as a combined oral contraceptive in South Africa (Steyn and Kluge, 2010), and contains 3 mg DRSP in combination with either a low dose (20 μg) EE (Yaz®) (Tan and Ediriweera, 2009) or a higher dose of 30 μg (Yasmin®) (Parsey and Pong, 2000). Interestingly, progestins have also been investigated in male contraception (Kamischke *et al.*, 2000, 2001; Nieschlag *et al.*, 2003; Gu *et al.*, 2004; Ilani *et al.*, 2012; Sitruk-Ware *et al.*, 2013). Here, progestins like MPA and NET-EN are administered in combination with testosterone, to reduce the secretion of gonadotropins and suppress sperm production (Kamischke *et al.*, 2000, 2001; Nieschlag *et al.*, 2003; Gu *et al.*, 2004; Ilani *et al.*, 2012; Sitruk-Ware *et al.*, 2013).

1.2.1.2   Hormone therapy (HT)

HT is used by millions of women to alleviate the symptoms associated with menopausal transition caused by a decline in estrogen levels (Greendale *et al.*, 1999; Hickey *et al.*, 2005). These symptoms include hot flushes, night sweats, bone loss, as well as vaginal atrophy and dryness (Greendale *et al.*, 1999; Hickey *et al.*, 2005). HT is administered as either estrogen alone to women who have had a hysterectomy, or estrogen in combination with a progestin to women with an intact uterus (Greendale *et al.*, 1999; Hickey *et al.*, 2012). The progestin component in HT serves to oppose the proliferative effects of estrogen on the endometrium, thereby preventing endometrial cancer (Whitehead *et al.*, 1979; Greendale *et al.*, 1999; Hickey *et al.*, 2005). Progestins can be administered with estrogens like E_2, EE, conjugated equine estrogen (CEE) or E_2-valerate (E_2V) (Mueck and Römer, 2018). Although the most common route of menopausal HT delivery is oral (Stanczyk *et al.*, 2013), it can also be delivered via intrauterine systems, transdermal patches, gels or sprays (Kuhl, 2005; Sitruk-Ware, 2007; Mueck and Römer, 2018).

MPA is commercially available as Prempro®, which contains MPA (1.5-5 mg) plus conjugated equine estrogen (CEE) (0.3-0.625 mg) (Prempro® package insert, Wyeth Pharmaceuticals Inc.), or the progestin-only Provera® (2.5-10 mg) (Provera® package insert, Pfizer Inc.) that is orally administered in combination with an estrogen-only HT (Nachtigall *et al.*, 1979). Peak serum concentrations of MPA have been reported to range between 0.2 and 13 nM (Hiroi *et al.*, 1975). NET-

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A (0.1–1 mg) can be administered orally in combination with either EE (0.0025-0.01 mg), available as FemHRT® (FemHRT® package insert, Warner Chicott Inc.), or E_2 (0.5–1 mg), available as either Avtivella® (Activella package insert, Movo Nordisk Inc.) or Mimvey® (Mimvey® package insert, Teva Pharmaceuticals Inc.). Alternatively, NET-A is also used in transdermal patches (Combipatch®) containing a fixed dose of E_2 (0.05 mg) in combination with NET-A (0.14–0.25 mg). Peak serum concentrations of NET-A in HT have been reported to be between 3.64-17.7 nM (Jinteli package insert, Teva Pharmaceuticals, USA). Although LNG (0.015 mg) can be administered via a transdermal patch containing E_2 (0.045 mg) (Climara Pro®) (Shulman *et al.*, 2002), it is frequently administered via an intrauterine device, such as the Mirena®, at lower doses than those used in contraception, and combined with either oral or transdermal estrogens like E_2 or E_2V (Suhonen *et al.*, 1997; Varila *et al.*, 2001; Raudaskoski *et al.*, 2002; Sturdee *et al.*, 2004; Varma *et al.*, 2006). Peak serum concentrations of LNG in HT have been reported to be within the range of 0.35–1.53 nM (Mirena® package insert, Bayer Health Care Pharmaceuticals Inc.). The fourth generation DRSP is commonly administered as an oral contraceptive pill that contains E_2 (1 mg E_2 plus 1–3 mg DRSP), and is commercially available as Angeliq® (Archer *et al.*, 2005; Genazzani *et al.*, 2013; Perkins *et al.*, 2018). Interestingly, peak serum concentrations of DRSP are reported to be higher (5.45–231.93 nM) than that of MPA, NET-A and LNG (Angeliq® package insert, Bayer Pharmaceuticals, Europe).

1.2.2 Side-effects

A number of side-effects have been associated with the clinical use of progestins, some more severe than others. In contrast, no major side-effects have been associated with the clinical use of P_4 (Writing group for the PEPI trial 1995; Fournier *et al.* 2008). Some of the side-effects considered to be less severe include headaches, nausea, mood changes, insomnia, reduced libido, breast tenderness, as well as irregular bleeding (Greydanus *et al.*, 2001; Sitruk-Ware, 2004, 2006; Erkkola and Landgren, 2005; Williams and Creighton, 2012). On the other hand, more severe side-effects include an increased risk of cardiovascular disease, VTE, genital tract infections, as well as ovarian and breast cancer (Writing group for the PEPI trial 1995; Rossouw *et al.* 2002; Beral *et al.* 2003; Gomes & Deitcher 2004; Fournier *et al.* 2008; Polis *et al.* 2016). In terms of progestins and the link to increased breast cancer, studies have shown that the postmenopausal use of MPA (Rossouw *et al.*, 2002; Beral *et al.*, 2003; Fournier *et al.*, 2008), NET, LNG and NOMAC (Beral *et al.*, 2003; Fournier *et al.*, 2008) is associated with an increased risk of invasive breast cancer. However, the association between many other progestins and increased breast cancer risk is unknown.

Most side-effects associated with the use of progestins are thought to be due to their cross-reactivity with steroid receptors other than the PR (Hapgood *et al.* 2004; Africander *et al.* 2011a; Stanczyk *et*

al. 2013). For example, the use of MPA, but not NET, has been shown to increase inflammation in the female genital tract due to its glucocorticoid activity . Increased inflammation in the genital tract has previously been shown to increase a woman's susceptibility to genital tract infections such as gonorrhea, *Chlamydia*, herpes simplex virus type (HSV)-2 and human immunodeficiency virus (HIV)-1 (Mostad *et al.*, 2000; Morrison *et al.*, 2004, 2010). Another side-effect associated with the glucocorticoid-like activity of MPA is decreased bone mineral density in adolescent women (Kass-Wolff, 2001; Cromer *et al.*, 2004; Williams and Creighton, 2012). However, these effects are reversed when usage is terminated (Cundy *et al.*, 1994).

Progestins from all generations have been associated with an increased risk of VTE, a condition referring to the formation of blood clots, which could subsequently lead to a stroke or pulmonary embolism (Odlind *et al.*, 2002; Rossouw *et al.*, 2002; Warren, 2004; Lidegaard *et al.*, 2009, 2012; Van Hylckama Vlieg *et al.*, 2009; Manzoli *et al.*, 2012; Sidney *et al.*, 2013). Interestingly, the fourth generation progestin, DRSP, is generally considered to be associated with a greater VTE risk than the third generation progestin, GES, which has a greater risk than first generation NET or second generation LNG (Kemmeren *et al.*, 2001; Odlind *et al.*, 2002; Van Vliet *et al.*, 2004; Lidegaard *et al.*, 2009; Van Hylckama Vlieg *et al.*, 2009).

The lack of anti-mineralocorticoid activity of progestins like NET-A and LNG (Oelkers, 2005; Stanczyk *et al.*, 2013) has been shown to lead to the elevation of blood pressure in postmenopausal women (reviewed in (Africander *et al.* 2011a)). In contrast, the fourth generation progestin, DRSP, which is known to display potent anti-mineralocorticoid activity, has been reported to elicit antihypertensive and cardio protective effects (reviewed in Oelkers, 2005). The androgenic activity of progestins like MPA, NET-A and LNG, has been shown to counteract the beneficial effects of estrogen on high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in postmenopausal women using HT (Crook *et al*. 1992; Writing Group for the PEPI trial, 1995). Thus, the use of progestins displaying androgenic activity could be an arterial risk factor causing cardiovascular disease (reviewed in Sitruk-Ware, 2000). In contrast, detrimental side-effects on lipoprotein levels have not been observed with progestins like NOMAC and DRSP, which display anti-androgenic activity (Ottosson *et al*. 1985; Saarikoski *et al*. 1990; Basdevant *et al*. 1991; Writing Group for the PEPI trial, 1995; Hulley *et al*. 1998; Sitruk-Ware 2000; Taneepanichskul & Phupong 2007; Ågren *et al*. 2011; Sitruk-Ware & Nath 2013).

1.3 Progestogens and breast cancer

1.3.1 Clinical and epidemiological studies

Breast cancer is the common cancer among women worldwide, and is the main cause of cancer-related deaths in women living in developed countries (Ferlay *et al.*, 2015; Torre *et al.*, 2015; Siegel *et al.*, 2016). While several investigations prior to 2002 have associated the use of progestins in HT with an increased risk of invasive breast cancer (Writing group for the PEPI trial 1995; Magnusson *et al.* 1999; Persson *et al.* 1999; Schairer *et al.* 2000; Ross *et al.* 2000), it was the findings of the Women's Health Initiative (WHI) trial (Rossouw *et al.*, 2002) that raised major concerns and confusion about the safety of HT. The WHI trial consisted of an estrogen-only arm where postmenopausal women who have undergone a hysterectomy were treated with CEE, and an estrogen-progestin arm, where postmenopausal women with an intact uterus were treated with CEE plus MPA. The latter arm was prematurely terminated in 2002 due to the incidence of increased invasive breast cancer and cardiovascular events, while the estrogen-only arm was terminated two years later due to increased risk of VTE and stroke, but not breast cancer risk. These results indicated that the progestin component in HT was responsible for the increased breast cancer risk. However, careful analysis of the trial data indicated that this increased risk of invasive breast cancer was only in women who had used HT for a prolonged period of time (5-10 years) (Sitruk-Ware and Plu-Bureau, 2004; Warren, 2004; Hickey *et al.*, 2005). Women who were first-time users of CEE plus MPA did not show this risk of invasive breast cancer (Sitruk-Ware and Plu-Bureau, 2004).

Increased breast cancer risk with the clinical use of progestins was, however, also shown by the Million Women Study (MWS) (Beral *et al.*, 2003). This study investigated multiple progestins and included women between the ages of 50 and 64 who had never used HT (never-users), women who were using HT at recruitment (current-users), and women who had previously used HT (past-users). MPA, NET and LNG increased risk in both current and past users, but not in never-users. Interestingly, there were no differences in breast cancer risk between the specific progestins investigated. In contrast to the WHI trial, the MWS showed that the estrogen-only arm also increased breast cancer risk, but that the risk was significantly less than the estrogen-progestin arm. Interestingly, results indicated a risk of breast cancer in overall HT users, in comparison to non-users, after only one year of HT usage (Beral *et al.* 2003; Sitruk-Ware & Plu-Bureau 2004), suggesting that HT activates existing tumours rather than inducing the formation of new tumours (Sitruk-Ware and Plu-Bureau, 2004; Hickey *et al.*, 2005). The French E3N cohort also showed that both estrogen-only and estrogen-progestin combination HT increased breast cancer risk in postmenopausal women

(Fournier *et al.*, 2008; Clavel-Chapelon, 2015). However, the use of estrogen-progestin combination HT was again associated with a larger risk than estrogen-only treatment. The progestins used in this study were MPA, NET-A and NOMAC, and no significant difference in breast cancer risk was observed between these progestins. Furthermore, results from this study showed that the risk of breast cancer increases with the duration of estrogen-progestin combination HT usage, even after as little as two years. In contrast to the above-mentioned studies , the Heart and Estrogen/Progestin Replacement Study (HERS) I and HERS II (Hulley *et al.*, 2002) studies reported no increased breast cancer risk with either the use of CEE or CEE plus MPA. Similarly, several studies have reported no increased breast cancer risk associated with the menopausal HT use of oral E_2-only or E_2 plus NET-A (Obel *et al.*, 1993; Tierney *et al.*, 2009; Schierbeck *et al.*, 2012). Moreover, is noteworthy that the French E3N study reported that the use of P_4 was not associated with an increased breast cancer risk (Fournier *et al.*, 2008). Similarly, results from the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial also reported that the use of oral micronized P_4 in combination with CEE does not increase breast cancer risk (Writing group for the PEPI trial 1995). In contrast, the Early Versus Late Intervention Trial (ELITE) showed that the use of a P_4 vaginal gel in combination with oral E_2 increased breast cancer risk (reviewed in (Marjoribanks *et al*. 2017)). Similarly, results from the Kronos Early Estrogen Prevention Study (KEEPS) showed that the use of micronized P_4 in combination with E_2 also increased breast cancer risk (reviewed in (Marjoribanks *et al*. 2017)).

Contradictory findings have also been reported for the use of progestins in contraception (Lee *et al.*, 1987; Althuis *et al.*, 2003; Hunter *et al.*, 2010; Li *et al.*, 2012; Beaber *et al.*, 2014). For example, some studies suggest that the contraceptive use of MPA as an intramuscular injection does not increase breast cancer risk (Kaunitz, 1996; Dills and Schreiman, 2003; Bakry *et al.*, 2008), while others have shown that it increased breast cancer risk in women between 20 and 58 years of age (Lee *et al.*, 1987; Li *et al.*, 2012). Similarly, the use of NET in combined oral contraceptives has been associated with an increased breast cancer risk (Althuis *et al.*, 2003), while another study reported no increase (Hunter *et al.*, 2010). Studies investigating the use of the second generation progestin, LNG, in combined oral contraception also reported an increased breast cancer risk (Althuis *et al.*, 2003; Hunter *et al.*, 2010). A recent population-based case-control study also showed that the use of NET, LNG and DRSP in combined oral contraceptives, increased breast cancer risk (Beaber *et al.*, 2014). Taken together, the evidence in the literature indicates that the role of progestins in breast cancer development is not straightforward. Further investigation is therefore required to determine the conditions under which progestins (in contraceptive or menopausal HT use) facilitate the development of breast cancer in women. Moreover, as there are numerous progestins with different

structures available for clinical use, it is important that individual progestins are assessed in terms of their risk profile.

1.3.2   Experimental studies

Numerous studies have investigated the effects of progestins on proliferation of normal and cancerous breast cell lines, but results are often conflicting (Jeng and Jordan, 1991; Van Der Burg *et al.*, 1992; Jeng *et al.*, 1992; Catherino *et al.*, 1993; Botella *et al.*, 1994; Kalkhoven *et al.*, 1994; Schoonen *et al.*, 1995a, 1995b; Franke and Vermes, 2003; Krämer *et al.*, 2006; Ruan *et al.*, 2012). A study by Krämer *et al.* (2006) showed that MPA increased the proliferation of growth factor-induced normal MCF10A breast cells, while P_4, NET and LNG had no effect. In the malignant HCC1500 breast cells, however, NET, LNG and GES enhanced the proliferation of growth factor-treated cells, while MPA had an inhibitory effect, and P_4 no significant effect (Krämer *et al.*, 2006). In agreement with this study, studies have shown that NET, LNG and GES increase proliferation of the human MCF-7 and T47D breast cancer cell lines (Jeng and Jordan, 1991; Jeng *et al.*, 1992; Van Der Burg *et al.*, 1992; Catherino *et al.*, 1993; Kalkhoven *et al.*, 1994; Schoonen *et al.*, 1995a; Franke and Vermes, 2003; Ruan *et al.*, 2012). (Schoonen *et al.*, 1995b). In contrast to these studies, a study by Botella *et al.* (1994) showed that MPA and NET decreased proliferation of T47D breast cancer cells in a dose-dependent manner. Numerous other studies have however showed that MPA has no effect on the proliferation of MCF-7 cells (Jeng and Jordan, 1991; Jeng *et al.*, 1992; Van Der Burg *et al.*, 1992; Catherino *et al.*, 1993; Schoonen *et al.*, 1995a; Ruan *et al.*, 2012). In terms of the fourth generation progestins, DRSP has been shown to increase proliferation of MCF-7 cells, while NOMAC had no effect (Ruan *et al.*, 2012). In contrast, NOMAC has previously been shown to decrease proliferation of T47D breast cancer cells in a dose-dependent manner (Botella *et al.*, 1994). Studies investigating the effects of P_4 on breast cancer cell proliferation are also conflicting. For example, P_4 has been shown to exert anti-proliferative (Formby and Wiley, 1998; Wiebe *et al.*, 2000) and proliferative (Schoonen *et al.*, 1995b; Franke and Vermes, 2003; Liang *et al.*, 2006) effects in the MCF-7 and T47D cells, but also no effect on MCF-7 breast cancer cell proliferation (Schoonen *et al.*, 1995a).

Studies investigating the effects of progestins on other hallmarks of breast cancer are limited. However, it has been shown that MPA elicits anti-apoptotic effects in the MCF-7, T47D and H466B breast cancer cell lines (Ory *et al.*, 2001; Franke and Vermes, 2003). Similarly, NET-A has been shown to inhibit apoptosis in the MCF-7 breast cancer cell line (Franke and Vermes, 2003). However, the effect of P_4 on apoptosis is contradictory. For example, at high concentrations (10 μM) P_4 is reported to elicit pro-apoptotic effects in MCF-7 (Franke and Vermes, 2003) and T47D cells (Formby and Wiley, 1998, 1999), while a lower concentration (100 nM) was shown to be anti-apoptotic in

T47D cells (Moore *et al.*, 2006). Furthermore, MPA, NES, DRSP and P$_4$, alone and in combination with E$_2$, enhanced migration and invasion of T47D breast cancer cells (Fu *et al.*, 2008). However, MPA was reported to promote migration and invasion to a larger extent than NES, DRSP and P$_4$. Furthermore, P$_4$, MPA, NET and LNG have been shown to increase angiogenesis, which promotes tumour growth and metastasis, in T47D cells via the upregulation of vascular endothelial growth factor (VEGF) mRNA expression (Hyder *et al.*, 1998; Mirkin *et al.*, 2005). P$_4$, MPA and NET also increased the protein expression of VEGF in T47D cells (Hyder *et al.*, 2001), while at least one study has shown that these progestins have no effect on VEGF protein expression in MCF-7, MDA-MB-231 and ZR-75 human breast cancer cells (Hyder *et al.*, 1998). Considering the above, it is clear that results from experimental studies investigating the effects of progestins on the hallmarks of breast cancer are not clear and warrants further investigation. Although *in vitro* studies cannot replace clinical studies, these experiments are useful in order to directly compare activities of different progestins in the same model systems, which may lay the foundation for future clinical studies.

1.4   Role of progestogens and steroid receptors in breast cancer biology

1.4.1   General mechanism of action of steroid receptors

Progestogens elicit their progestogenic effects by binding to the progesterone receptor (PR) (Hapgood *et al.* 2004; Sitruk-Ware 2006; Stanczyk *et al.* 2013). The PR, together with the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), comprise the steroid receptor family. These receptors are ligand-activated transcription factors that all share a high degree of structural similarity (Figure 1.2) (reviewed in (Griekspoor *et al.* 2007)). Although steroid receptors function via similar mechanisms, their target genes and target tissues may differ (McKenna and O'Malley, 2002). In general, these steroid receptors all contain a highly variable NH$_2$-terminal domain (NTD), a well conserved DNA-binding domain (DBD) and a moderately conserved ligand-binding domain (LBD) located at the COOH-terminal (Mangelsdorf *et al.*, 1995). While the LBD is involved in the binding of ligand to the steroid receptor, the DBD allows for binding to specific DNA sequences in target genes, steroid receptor dimerization, and interactions with co-factors (reviewed in (Griekspoor *et al.* 2007)). Furthermore, steroid receptors harbor two major transcriptional activation domains, namely activation function-1 (AF-1), which is constitutively active and located in the NTD, and activation function-2 (AF-2), which is a ligand-dependent domain located in the LBD (Griekspoor *et al.*, 2007). The AF-2 domain interacts with co-activators to induce the transcriptional activity of the receptor (Aagaard *et al.*, 2011), while the AF-1 domain allows for

optimal transcriptional activity of the steroid receptor (Lavery and McEwan, 2005; Aagaard *et al.*, 2011).

In the absence of hormone, the AR, GR and MR predominantly reside in the cytoplasm, while the ER subtypes and PR-A isoform are found predominantly in the nucleus (Li, 2005; Griekspoor *et al.*, 2007). PR-B is distributed uniformly between the cytoplasm and the nucleus (Griekspoor *et al.*, 2007). When a ligand binds to a steroid receptor, a conformational change is induced in the receptor, allowing it to dissociate from chaperone proteins, such as heat-shock protein (Hsp)90 and Hsp70, phosphoprotein p23, and immunophillin p59 (Pratt and Toft, 1997; Griekspoor *et al.*, 2007). The ligand-steroid receptor complex subsequently translocates to the nucleus, where it binds to specific regulatory elements within the promoter region of target genes to activate transcription (transactivation) (reviewed in (Huang *et al.* 2010; Africander *et al.* 2011a)). Transcription can also be repressed (transrepression) via the liganded steroid receptor interacting with DNA-bound transcription factors (reviewed in (Huang *et al.* 2010; Africander *et al.* 2011a)).

For transactivation of target genes, it is generally accepted that the ligand-bound steroid receptor binds as a dimer to specific palindromic DNA sequences known as steroid response elements (SREs) (reviewed in (Griekspoor *et al.* 2007)). Due to the high level of conservation in the DBD of steroid receptors (reviewed in (Africander *et al.* 2011a)), most steroid receptors can bind the same SRE. For example, while the PR binds to progesterone response elements (PREs), this consensus DNA sequence can also be bound by other steroid receptors like the AR, MR and GR. In this case, the SRE is then called the androgen response element (ARE), mineralocorticoid response element (MRE) and glucocorticoid response element (GRE), respectively. In contrast to the AR, MR and GR, the ER is unique in that it binds specifically to estrogen response elements (EREs) (Klinge, 2001). On the other hand, the primary mechanism for transrepression involves an indirect binding of the ligand-bound steroid receptor to DNA-bound transcription factors such as nuclear factor-kappa B (NFκB) or activator protein-1 (AP-1) (McEwan *et al.*, 1997; Webster and Cidlowski, 1999). Although not described here, it is noteworthy that steroid hormones can also elicit their effects via rapid non-genomic mechanisms. These mechanisms may involve membrane steroid receptors or the activation of other signaling pathways, like the c-Src/Ras/mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (Stellato, 2004; Boonyaratanakomkit *et al.*, 2007; Wieman, 2007; Zhu *et al.*, 2008; Grossmann and Gekle, 2009).

All the steroid receptors have been implicated in breast cancer biology, and often influence each other's activity (reviewed in (Sikora 2016; Perkins *et al.* 2018)). As the PR, ER and AR are the best

studied in breast cancer, and knowing that some progestins can act via these receptors, the following sections will briefly describe the role of progestins and/or the PR, ER and AR on the hallmarks of breast cancer.
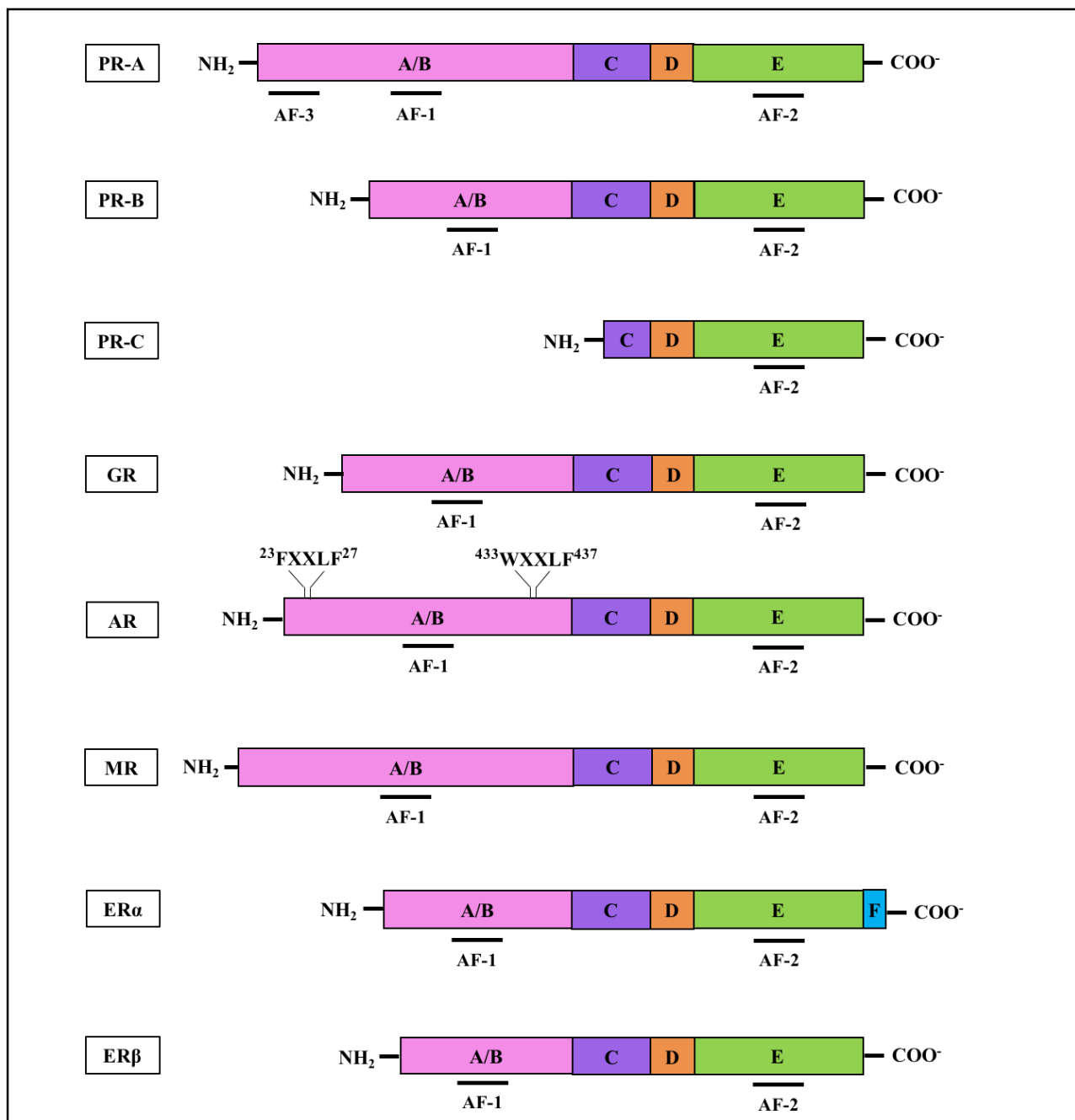
Figure 1.2. A schematic illustration of the general structural organization of the functional domains of steroid receptors. Steroid receptors contain a variable NH_2-terminal domain (NTD), denoted by A/B, with a constitutively active activation function-1 (AF-1) transactivation domain. The highly conserved DNA-binding domain (DBD) is denoted by C, while the hinge region is denoted by D. The relatively conserved ligand-binding domain (LBD), which contains a ligand-dependent AF-2 transactivation domain, is located at the COOH-terminal of the receptor (E). The additional AF-3 domain in PR-B is also shown. ERα contains an additional domain at the COOH-terminal (F), of which the function is not known. The relevant motifs indicated in the AF-1 domain of the AR are crucial for the N/C interaction.

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1.4.2   Progestogens and the PR isoforms

The biological effects of progesterone are mediated by the PR (Grimm *et al.*, 2016), which exists as three distinct isoforms (Figure 1.2), namely PR-A (94 kDa), PR-B (110 kDa) and PR-C (60 kDa), transcribed from different promoters of a single gene (Kastner *et al.*, 1990; Wei and Gonzalez-Aller, 1990; Rekawiecki *et al.*, 2011). PR-C, however, is not functional as it lacks a significant part of the DBD, which thus prevents DNA binding and transcription of target genes (Samalecos and Gellersen, 2008; Daniel *et al.*, 2011; Rekawiecki *et al.*, 2011; Abdel-Hafiz and Horwitz, 2014). PR-B, but not PR-A, contains an AF-3 domain in the NTD, which facilitates the binding of certain co-activators to PR-B (Giangrande *et al.*, 2000; Graham and Clarke, 2002; Tung *et al.*, 2006). Thus, PR-B is considered to be more transcriptionally active than PR-A in the presence of agonist (Kastner *et al.*, 1990; Edwards *et al.*, 1995; Rekawiecki *et al.*, 2011; Jacobsen and Horwitz, 2012). In the absence of ligand, however, PR-A is generally more transcriptionally active than PR-B (Jacobsen *et al.*, 2002). While PR-A and PR-B can regulate the same genes, there are some genes that are regulated only by a specific isoform (Richer *et al.*, 2002; Lanari *et al.*, 2012). For example, only 25% of P_4-regulated genes are regulated by both PR-A and PR-B, while nearly 65% are regulated only by PR-B and 4% are regulated only by PR-A (Richer *et al.*, 2002; Lanari *et al.*, 2012). It is thus not surprising that the biological roles of PR-B and PR-A have been shown to differ (Clarke and Sutherland, 1990; Conneely and Lydon, 2000; Conneely *et al.*, 2000, 2003; Graham and Clarke, 2002; Mulac-Jericevic and Conneely, 2004; Diep *et al.*, 2015). In breast cancer, for example, PR-B has been shown to mediate the P_4-induced upregulation of genes that promote metastasis, while PR-A mediated the upregulation of anti-apoptotic genes by P_4 in T47D breast cancer cells (Richer *et al.*, 2002). PR-B was also required for P_4-induced upregulation of VEGF protein expression, which promoted tumour growth and metastasis (Wu *et al.*, 2004). Considering that the PR isoforms differ in their transcriptional activity, as well as their biological roles, it is concerning that the role of the PR in breast cancer is generally studied as a whole, without consideration of the role of the individual isoforms.

PR-A and PR-B are expressed in equimolar ratios in the normal breast (Mote *et al.*, 2002; Richer *et al.*, 2002), while PR-A is often over-expressed in breast cancer (Graham *et al.*, 1995; Bamberger *et al.*, 2000; Ariga *et al.*, 2001; Hopp *et al.*, 2004). In fact, the ratio of PR-A to PR-B expression is an important determinant of breast cancer development and progression (Graham *et al.*, 1995; Mote *et al.*, 2002; Hopp *et al.*, 2004; Cui, 2005). For example, breast tumours expressing more PR-A relative to PR-B have been shown to be more aggressive, and lead to a poor disease-free survival rate (Hopp *et al.*, 2004). Considering that progestins are PR ligands (Sitruk-Ware, 2006), and that some progestins have been shown to increase breast cancer, the question that arises is whether their effects in breast cancer are via the PR. Although this is now a topic of ongoing research, most studies do not

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discern between the role of the PR isoforms. However, a limited number of studies have investigated the role of the PR isoforms in mediating the effects of progestins on breast cancer hallmarks (Wu *et al.*, 2004; Moore *et al.*, 2006; Giulianelli *et al.*, 2012; Bellance *et al.*, 2013; Kariagina *et al.*, 2013; Wargon *et al.*, 2015). For example, MPA has been shown to increase proliferation of the T47D human breast cancer cell line by upregulating the expression of cyclin D1 via the PR (Giulianelli *et al.*, 2012; Wargon *et al.*, 2015). Wargon and co-workers in fact showed that PR-B was required for the proliferative effects of MPA in T47D cells (Wargon *et al.*, 2015). Interestingly, ERα has also been shown to be required for the PR-B-mediated effects of MPA in T47D cells (Giulianelli *et al.*, 2012). This study was one of the first to show crosstalk between the PR and ERα in breast cancer. Promegestone (R5020), a PR-specific progestin widely used as an experimental tool to investigate the role of the PR, elicited anti-apoptotic effects in the MCF-7 and MDA-MB-231 breast cancer cell lines (Moore *et al.*, 2006). As the effects of R5020 were reversed by the antagonist, mifepristone (RU486), the authors suggested that the PR was involved in these anti-apoptotic effects (Moore *et al.*, 2006). Although the role of the individual PR isoforms was not investigated by Moore *et al.* (2006), it has previously been shown that both PR-A and PR-B contributed to the R5020-induced increase in migration and invasion of MDA-MB-231 breast cancer cells (Bellance *et al.*, 2013). In contrast to these findings, PR-B, but not PR-A, was required for the R5020-induced increase in invasion of the T47D breast cancer cell line (Kariagina *et al.*, 2013). Similarly, it was shown that both PR isoforms, but mainly PR-B, was required for the MPA- and NET-induced increase in VEGF protein expression in T47D cells (Wu *et al.*, 2004). Considering these contradictory findings, and the limited PR isoform-specific investigations, it is clear that more molecular studies are needed to directly compare PR isoform-specific effects of P_4 and progestins on hallmarks of breast cancer. Furthermore, as the ratio of PR-A to PR-B expression is a determining factor in breast cancer development and progression (Graham *et al.*, 1995; Mote *et al.*, 2002; Hopp *et al.*, 2004; Cui, 2005), it is important to investigate the influence of the ratio of PR-A to PR-B in progestogen effects on breast cancer.

1.4.3   Estrogens and the estrogen receptor (ER) subtypes

Estrogens are involved in the growth and differentiation of the breast and other sex organs of the female reproductive system (Gruber *et al.*, 2002; Gouglet *et al.*, 2005; Thomas and Potter, 2013). There are three types of estrogens which are produced in the female body, namely estrone (E_1), 17β-estradiol (E_2) and estriol (E_3) (Thomas and Potter, 2013; Samavat and Kurzer, 2015), with E_2 being the most biologically active (Samavat and Kurzer, 2015). The biological effects of estrogens are mediated by the ER, which exists as two subtypes (Figure 1.2), ERα (66 kDa) and ERβ (56 kDa), transcribed from different genes (Menasce *et al.*, 1993; Matthews and Gustafsson, 2003). ERα and

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ERβ share a high degree of sequence identity (96%) within their DBD, which explains why these subtypes can bind to similar sites in target genes (Matthews and Gustafsson, 2003). However, the LBD of ERα and ERβ is moderately conserved with a sequence identity of 53%, which could explain their differences in ligand binding specificity (Mosselman *et al.*, 1996; Pearce and Jordan, 2004).

It is well-known that E_2 and ERα are considered the main etiological factors in breast cancer (Vogel, 2018). In fact, ERα is expressed in nearly 70% of all breast cancers (Jensen and Jordan, 2003; Pearce and Jordan, 2004; Conzen, 2008), and is crucial for the proliferative effects of E_2 in breast cancer (Couse & Korach 1999; Brisken & O'Malley 2010). This was shown in an animal experiment where exposure of ERα-knockout mice to E_2 did not result in the formation of breast cancer tumours (Couse & Korach 1999; Brisken & O'Malley 2010). The actions of the E_2-bound ERα in breast cancer include dysregulation of the cell cycle and apoptotic pathway, which ultimately promotes tumorigenesis (Hartman *et al.*, 2009). For example, the E_2-bound ERα has been shown to upregulate cell cycle genes, such as c-myc, cyclin D1, and cyclin A2, as well as anti-apoptotic genes, such as Bcl-x (Altucci *et al.*, 1996; Frasor *et al.*, 2003; Helguero *et al.*, 2005; Hartman *et al.*, 2009; Welboren *et al.*, 2009). In addition, pro-apoptotic genes, such as Bax, have been shown to be downregulated by E_2 (Helguero *et al.*, 2005). Moreover, E_2 has also been shown to stimulate the invasive and metastatic potential of MCF-7 ERα-positive human breast cancer cells (Shafie and Liotta, 1980; Osborne *et al.*, 1985; Cos *et al.*, 1986). It is thus not surprising that current therapies for E_2-dependent breast cancer either inhibit ERα activity or inhibit E_2 synthesis (Lanari *et al.*, 2012; Lim *et al.*, 2016; Doan *et al.*, 2017). These therapies include selective ER modulators (SERMs), such as tamoxifen, selective ER downregulators (SERDs), such as fulvestrant (ICI 182,780) and aromatase inhibitors (AIs) (Jordan, 2007; Mirzaie *et al.*, 2013; Nantasenamat *et al.*, 2013; Akçay and Bayrak, 2014; Patani and Martin, 2014). SERMs are ER antagonists that compete with E_2 and modulate ER activity, while SERDSs are ER antagonists that induce ER degradation (reviewed in (Patel & Bihani 2018)).

Immunohistochemical analysis has shown that both ERα and ERβ are expressed in the normal breast, with the expression of ERβ being higher than that of ERα (Clarke *et al.*, 1997; Russo *et al.*, 1999; Speirs *et al.*, 2004). In breast cancer, the subtypes are co-expressed in approximately 59% of primary breast tumours (Murphy *et al.*, 2003), but expression of ERα is higher than that of ERβ (Leygue *et al.*, 1998; Roger *et al.*, 2001; Murphy *et al.*, 2003; Shaaban *et al.*, 2003). While ERα promotes tumorigenesis, ERβ antagonizes ERα-mediated effects (Leygue *et al.*, 1998; Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999; Zhao *et al.*, 2008). However, the role of ERβ in breast cancer is bi-faceted, as it mimics the proliferative effects of ERα in ER-negative breast cancer (Leygue *et al.*, 1998; Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999; Williams *et al.*, 2008; Zhao *et al.*, 2008; Hartman *et al.*, 2009; Leygue

and Murphy, 2013). For example, ERβ has been shown to increase breast cancer cell growth by upregulating the expression of proliferative markers like Ki67 and cyclin A, and downregulating the expression of the tumour-suppressor gene, p21 (Moore *et al.*, 1988; Hou *et al.*, 2004; O'Neill *et al.*, 2004; Skliris *et al.*, 2006; Fox *et al.*, 2008). Furthermore, it has been shown that an increase in ERβ expression, by stably transfecting different amounts of ERβ, resulted in an increased rate of proliferation of the ERα-negative MDA-MB-231 and MDA-MB-453 breast cancer cells (Tonetti *et al.*, 2003; Hou *et al.*, 2004). Thus, in contrast to the inhibitory role of ERβ in ERα-positive breast cancer, the role of ERβ in ERα-negative breast cancer is not considered favorable (Tonetti *et al.*, 2003; Hou *et al.*, 2004; Leygue and Murphy, 2013).

From the above, it is clear that the role of E_2 and the ER subtypes in breast cancer is complex. An added complexity is the fact that crosstalk between ERα and the PR plays an important role in breast cancer pathogenesis (Giulianelli *et al.*, 2012; Daniel *et al.*, 2015; Mohammed *et al.*, 2015; Singhal *et al.*, 2016). For example, it has been shown that unliganded PR-B can modulate the transcriptional activity of ERα by forming ERα/PR-B complexes at the promoters of certain ER target genes (Daniel *et al.*, 2015). This resulted in a more aggressive proliferative response upon E_2 stimulation of the MCF-7 breast cancer cell line (Daniel *et al.*, 2015). In contrast, the P_4- and R5020-activated PR has been shown to redirect the chromatin binding of the E_2-activated ERα to PR binding sites (Mohammed *et al.*, 2015). This involved an association between the PR and ERα, and ultimately resulted in altered transcriptional activity of ERα and the inhibition of cell growth in both MCF-7 BUS and T47D cell lines (Mohammed *et al.*, 2015). A similar mechanism was subsequently reported in primary ERα- and PR-positive breast cancer tumours (Singhal *et al.*, 2016). A very recent study highlighted the PR isoform-specific modulation of ERα transcriptional activity (Singhal *et al.*, 2018). It showed that the inhibition of gene expression and ER chromatin binding in T47D breast cancer cells was greater in the presence of PR-A than PR-B (Singhal *et al.*, 2018). Moreover, the study by Singhal and co-workers also showed that the repression of ERα transcriptional activity is greater when the PR is bound to antagonist, than when it is bound to (Singhal *et al.* 2018). Taken together, these results suggest that the interplay between ERα and the PR can be associated with either good or poor prognosis in breast cancer, and that the outcome may be determined by the absence or presence of PR ligands. However, as progestins like MPA, NET-A and LNG are associated with increased breast cancer, we would hypothesize that not all PR ligands would result in a good prognosis. Further investigations are thus required to elucidate the role of the interplay between ERα and the PR. Moreover, whether similar crosstalk exists between the PR and ERβ is not known. Understanding the role of ER-PR crosstalk in breast cancer, as well as whether this interplay is

responsible for the observed effects of progestins in breast cancer, may facilitate the design of new progestins that do not cause increased breast cancer.

1.4.4 Androgens and the androgen receptor (AR)

A number of studies have shown that androgens and the AR play important roles in breast cancer biology (reviewed in (Rahim & O'Regan 2017)). In the presence of a classical AR agonist, an interaction between the amino- and carboxyl-terminal of the AR, more commonly known as the N/C interaction, occurs (He *et al.*, 1999, 2000; Kemppainen *et al.*, 1999; Schaufele *et al.*, 2005; Van Royen *et al.*, 2007, 2012; Africander *et al.*, 2014). This AR N/C interaction is mediated by the AF-1 domain of the NTD and the AF-2 domain of the LBD (He *et al.*, 1999, 2000). More specifically, the subdomains of AF-1 that have been implicated in this interaction include the FXXLF motif (sequence ^{23}FQNLF^{27}) and WXXLF motif (sequence ^{433}WHTLF^{437}) (Figure 1.2) (He *et al.*, 2000).The AR N/C interaction promotes stabilization of the receptor and slows down the rate of ligand dissociation (He *et al.*, 1999; Kemppainen *et al.*, 1999). The fact that the AR N/C interaction is not induced by all AR agonists, indicates that the interaction is not imperative to render the AR transcriptionally active (Kemppainen *et al.*, 1999; Africander *et al.*, 2014). Interestingly, the AR N/C interaction can take place either within one molecule (intramolecular) or between two AR molecules (intermolecular) (Schaufele *et al.*, 2005), and is lost upon DNA binding, subsequently allowing the interaction of co-factors and basal transcription machinery (Van Royen *et al.*, 2007, 2012).

The AR is expressed in approximately 90% of all primary breast tumours (Søreide *et al.*, 1992; Garay and Park, 2012). It is thus not surprising that AR-targeted breast cancer treatment is actively being investigated (reviewed in (Rahim & O'Regan 2017)). However, the precise role and prognostic effect of the AR in breast cancer is greatly dependent on whether ERα is expressed (Fioretti *et al.*, 2014; Lim *et al.*, 2014). While the AR has been associated with an anti-proliferative role and favorable prognosis in ERα-positive breast cancer (Castellano *et al.*, 2010; Hu *et al.*, 2011; Park *et al.*, 2011; Tsang *et al.*, 2014), it is associated with a poor prognosis when mimicking the proliferative effects of ERα in ERα-negative breast cancers (Robinson *et al.*, 2011; Rahim and O'Regan, 2017). For example, it has been shown that the natural androgen DHT and the synthetic non-metabolizable androgen, methyltrienolone (R1881), increased proliferation of the ERα-negative MDA-MB-453 breast cancer cell line, as well as MDA-MB-453 xenografts via the AR (Doane *et al.*, 2006; Cochrane *et al.*, 2014; Narayanan *et al.*, 2014). Moreover, the AR has been shown to elicit growth promoting effects in molecular apocrine breast cancer tumours, which are ERα-negative and HER2-positive (Ni *et al.*, 2011). Anti-androgens, like bicalutamide and enzalutamide, are thus currently being

investigated as options for treatment of AR-positive triple negative breast cancer (TNBC) (reviewed in (Rahim & O'Regan 2017)).

In ERα-positive breast cancer, however, the favorable prognosis of the AR may be due to the inhibition of ERα activity (Birrell *et al*. 1995b; Panet-Raymond *et al*. 2000; Greeve *et al*. 2004; Peters *et al*. 2009; Need *et al*. 2012; Perkins *et al*. 2018). At least two mechanisms have been reported for the inhibitory role of the AR in ER-positive breast cancer (Peters *et al.*, 2009; Rizza *et al.*, 2014). For example, the DHT-bound AR has been shown to compete with ERα for binding to EREs in ER target genes (Peters *et al.*, 2009). Another study has shown that crosstalk between the AR and ERβ may also be involved in the inhibitory effect of the AR, as both DHT and the synthetic androgen, Mibolerone (Mib), increased ERβ mRNA and protein expression (Rizza *et al.*, 2014). The mechanism for this increase in ERβ expression was due to the activated AR binding to an ARE in the ERβ gene promoter (Rizza *et al.*, 2014). Moreover, this increase in ERβ expression ultimately resulted in the inhibition of breast cancer cell growth in both the MCF-7 and ZR-75 breast cancer cell lines (Rizza *et al.*, 2014). However, this study did not investigate the effects on ERα expression. Considering that the ER subtypes have opposite effects in breast cancer cell proliferation, with ERα being the driver of breast cancer cell proliferation (Leygue *et al.*, 1998; Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999; Zhao *et al.*, 2008), it is important that its expression also be investigated. It is noteworthy that clinical trials are currently investigating the use of selective AR modulators (SARMs) for the treatment of ERα-positive breast cancer (reviewed in (Rahim & O'Regan 2017)). SARMs are AR agonists that activate the AR in a tissue-specific manner (Narayanan *et al.*, 2018). Interestingly, MPA, which is a potent AR agonist (Africander *et al*. 2014; Louw-du Toit *et al*. 2017a), has been used in breast cancer treatment at doses higher than those found in menopausal HT (Birrell *et al*. 1995a; Carroll *et al*. 2016). In fact, the effects of MPA as breast cancer therapy has been shown to require the AR at the molecular level (Birrell *et al*. 1995a). Furthermore, like DHT, MPA also inhibited ERα transcriptional activity via the AR in MDA-MB-231 breast cancer cells, albeit to different extents (Peters *et al.*, 2009). However, it has also been shown that the AR-mediated effects of MPA disrupts the normal signaling of androgens which play a protective role in breast cancer (Birrell *et al.*, 2007). This indicates that the androgenic actions of MPA in breast cancer is not straightforward. Like MPA, progestins such as NET-A, LNG and GES, have also been shown to be potent AR agonists (Africander *et al*. 2014; Louw-du Toit *et al*. 2017a). Whether the progestins displaying androgenic properties similar to DHT would increase ERβ expression, is not known. Furthermore, whether androgens and/or androgenic progestins would regulate ERα expression is also not known, and warrants further investigation.

1.5   Conclusion

Considering that breast cancer is the most commonly diagnosed cancer among women worldwide (Ferlay *et al.*, 2015; Torre *et al.*, 2015; Siegel *et al.*, 2016), it is important to understand the underlying mechanisms so as to facilitate the design of new drugs for the treatment and prevention of breast cancer. All members of the steroid receptor family have been implicated in breast cancer pathogenesis, and often these steroid receptors influence each other's activity (reviewed in (Sikora 2016; Perkins *et al.* 2018)). For example, the AR is expressed in the majority of ERα-positive breast cancers (Agoff *et al.*, 2003; Niemeier *et al.*, 2010; Tsang *et al.*, 2014), and has been associated with a growth-inhibitory role (Castellano *et al.*, 2010; Hu *et al.*, 2011; Park *et al.*, 2011; Tsang *et al.*, 2014). Several mechanisms have been proposed for this inhibition, which all involve crosstalk of the AR with the ER subtypes (Figure 1.3) (Peters *et al.*, 2009; Rizza *et al.*, 2014). One mechanism involves the DHT-activated AR inhibiting the transcriptional activity of ERα (Peters *et al.*, 2009). Interestingly, activation of the AR by MPA, also resulted in this inhibition of ERα activity (Peters *et al.*, 2009). Another mechanism involves the Mib- and DHT-activated AR increasing ERβ mRNA and protein expression (Rizza *et al.*, 2014). Considering that MPA, NET-A and LNG display similar androgenic properties as DHT (Africander *et al.* 2014; Louw-du Toit *et al.* 2017a), and that they have previously been associated with increased breast cancer risk (Rossouw *et al.*, 2002; Beral *et al.*, 2003; Fournier *et al.*, 2008), the question arises whether these progestins would also increase ERβ expression via an AR-mediated mechanism. Moreover, as ERβ and ERα play opposing roles in breast cancer, it is important to also assess AR-mediated effects of the androgens and progestins on ERα expression. In view of the wide-spread expression of the AR in ER-positive breast cancer (Søreide *et al.*, 1992; Garay and Park, 2012), it is not surprising that AR-targeted breast cancer treatment is actively being researched (reviewed in (Rahim & O'Regan 2017)). In fact, MPA has been used in breast cancer treatment, and the AR has been shown to be required for its effects (Birrell *et al.* 1995a). Considering the above, and the fact that the role of progestins in breast cancer is not straightforward, it is crucial to investigate the AR-mediated effects of progestins binding to the AR.
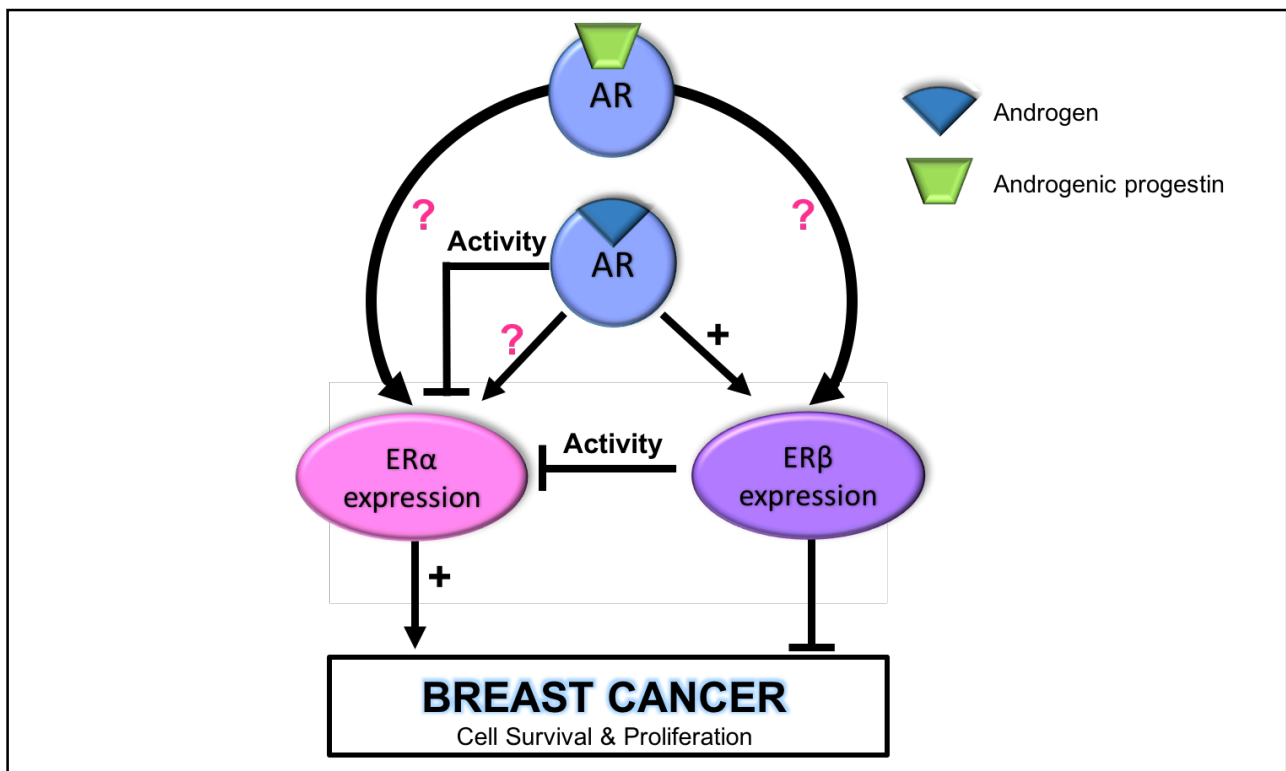
Figure 1.3. Interplay between the AR and ER subtypes may lead to positive effects on breast cancer. ERα promotes cell survival and proliferation, while ERβ antagonizes ERα-mediated effects. In ER-positive breast cancer, the androgen-activated AR inhibits cell proliferation by inhibiting the activity of ERα and upregulating ERβ expression. Effects of the progestin-activated AR on the expression of both ERβ and ERα are unknown.

1.6   Hypothesis and aims of study

Rizza and co-workers have shown that activation of the AR by the natural androgen, DHT, and a potent synthetic non-metabolizable androgen, Mib, leads to the upregulation of ERβ expression (Rizza *et al.*, 2014). Whether these androgens elicit similar effects on ERα expression was not investigated. However, considering that ERα is associated with a proliferative role in breast cancer, and that anti-proliferative effects were observed when ERβ expression was increased, one would not expect to see increased ERα expression in the presence of these androgens. Some progestins used in contraception and menopausal HT can bind to the AR and display androgenic properties similar to DHT, while others display anti-androgenic properties similar to natural P_4. The primary hypothesis of this study was thus that the androgenic progestins, unlike P_4 and the anti-androgenic progestins, would increase the expression of ERβ, but not ERα, via a mechanism requiring the AR. In order to test the hypothesis, the aims of this study were as follows:

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1. To compare the potential of the progestins displaying androgenic and anti-androgenic activity to either induce the AR N/C interaction, or antagonize the DHT-induced AR N/C interaction, as a measure of conformational change and activation of the AR. Mammalian two-hybrid assays were performed in the COS-1 African monkey kidney fibroblast cell line.

2. To investigate whether progestins known to bind to the AR and display androgenic properties, would protect the AR protein from degradation. Western blot analysis was performed in the MCF-7 BUS breast cancer cell line.

3. To investigate the steroid receptor-mediated effects of androgens, P_4, as well as the androgenic- and anti-androgenic progestins on proliferation of the MCF-7 BUS and T47D breast cancer cells, using cell viability assays and steroid receptor-specific antagonists.

4. To directly compare the effects of androgens, P_4, as well as the androgenic- and anti-androgenic progestins on the mRNA and protein expression of ERβ and ERα in human breast cancer cell lines using real-time quantitative PCR and western blotting.

5. To investigate the role of the AR in the response in (4), using an AR antagonist.

Importantly, while the terms "we" and "our" are often used throughout this thesis, all experimental work was performed by the candidate.

Chapter 2

Materials and Methods

2.1   Test compounds

The compounds used in this study (Table 2.1) were mostly purchased from Sigma-Aldrich, South Africa, except mibolerone (Mib), which was purchased from Steraloids, United States of America (USA). Stock solutions of the test compounds were prepared in absolute ethanol (EtOH), and stored at −20˚C. For all assays, the test compounds were diluted 1000 times in serum-free culturing medium, so that the final concentration of EtOH was 0.1% (v/v). Thus, 0.1% (v/v) EtOH served as a vehicle control in all experiments.

Table 2.1. Agonists and antagonists used in this study.

			Common name	Chemical name	Abbreviation
Agonists	**Androgens**	**Natural**	Dihydrotestosterone	5α-androstan-17β-ol-3-one	DHT
		Synthetic	Mibolerone	17β-hydroxy-7α, 17α-dimethylestr-4-en-3-one	Mib
	Progestogens	**Natural**	Progesterone	4-pregnene-3, 20-dione	P_4
		Synthetic	Medroxy-progesterone acetate	6α-methyl-17α-hydroxy-progesterone acetate	MPA
			Norethisterone acetate	17α-ethynyl-19-nortesterone 17β-acetate	NET-A
			Levonorgestrel	17α-ethynyl-17β-hydroxy-18α-homoestr-4-en-3-one	LNG
			Gestodene	17α-13-ethyl-17-hydroxy-18,19-dinorpregna-5,15-dien-20-yn-3-one	GES
			Nesterone	17α-acetoxy-16-methylene-19-norprogesterone	NES
			Nomegestrol acetate	17α-acetoxy-6-methyl-6-dehydro-19-norprogesterone	NOMAC
			Drospirenone	6β,7β:15β,16β-dimethylenespirolactone	DRSP
			Promegestone	(17β)-17-methyl-17-propanoylestra-4,9-dien-3-one	R5020
	Estrogens	**Natural**	17β-Estradiol	17β-estra-1, 3, 5 (10)-triene-3, 17-diol	E_2
	Gluco-corticoids	**Synthetic**	Dexamethasone	(11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	Dex
Antagonists		**AR**	Hydroxyflutamide	2-hydroxy-2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propanamide	OHF
			Bicalutamide	N-(4-cyano-3-(trifluoromethyl)phenyl)-3-((4-fluorophenyl)sulfonyl)-2-hydroxy-2-methylpropanamide	Bical
		ER	Fulvestrant ICI 182,780	7α-(9-(4,4,5,5,5-pentafluoropentylsulfinyl)-nonyl)estra-1,3,5(10)-triene-3,17β-diol	ICI
		PR/GR/AR	Mifepristone	11β-[4-(dimethylamino)-phenyl]-17β-hydroxy-17α-(prop-1-yn-1-yl)estra-4,9-dien-3-one	RU486

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2.2   Cell culture

The T47D human breast cancer cell line was a kind gift from Prof. I. Parker (University of Cape Town, South Africa), while the COS-1 African monkey kidney fibroblast cells and HEK-293 human embryonic kidney cells, were obtained from the American Type Culture Collection (ATCC) (USA). All these cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing phenol-red and 4.5 g/mL glucose (Sigma-Aldrich, South Africa), supplemented with 10% (v/v) fetal calf serum (FCS) (Separations, South Africa), 100 IU/mL penicillin and 100 μg/mL streptomycin (PenStrep) (Sigma-Aldrich, South Africa). The MDA-MB-453 triple negative human breast cancer cell line, a kind gift from Prof. A. Edkins (Rhodes University, South Africa), was maintained in the same medium as above, with the addition of 2 mM L-glutamine (Sigma-Aldrich, South Africa). The MCF-7 BUS human breast cancer cell line, obtained from Prof. A. Soto (Tufts University, Massachusetts), was maintained in DMEM containing phenol-red and 4.5 g/mL glucose, supplemented with 5% (v/v) heat-inactivated FCS (Addendum A) and PenStrep. All experiments were conducted within the first 35 passages since the cells were thawed from storage. Cells were tested regularly for mycoplasma contamination by means of Hoechst staining (Freshney *et al.*, 1987), and only mycoplasma negative cell lines were used in experiments. Representative mycoplasma negative images of the cell lines used in this study are shown in Figure B1 (Addendum B). All cell lines were maintained in 75 cm^2 culture flasks (Bio-Smart Scientific, South Africa) and incubated at 37˚C in 90% humidity and 5% CO_2.

2.3   Plasmids

The cDNA expression vectors for the human wild-type AR DBD-LBD (pSG5-hAR (DBD-LBD)), and the human AR NTD-VP16 fusion protein (pSNATCH-II-hAR (NTD)), were obtained from Prof. F. Claessens (University of Leuven, Belgium) (Alen *et al.*, 1999). The luciferase promoter-reporter construct, pTAT-GRE-E1b-luc, which is driven by the E1b promoter and contains two copies of the rat tyrosine aminotransferase (TAT)-GRE, was provided by Prof. G. Jenster (Erasmus University of Rotterdam, Netherlands) (Jenster *et al.*, 1997). Plasmids expressing the human PR-A and PR-B isoforms (pSG5-hPR-A and pSG5-hPR-B), were obtained from Prof. E. Kalkhoven (University of Utrecht, Netherlands) (Kalkhoven *et al.*, 1996), while the human wild-type AR (pSG5-hAR) was a kind gift from Dr. H. Klocker (Medical University of Innsbruck, Austria) (Schneikert *et al.*, 1996). The plasmid expressing the human GR (pRS-hGRα) was obtained from Prof. R. Evans (Harvard Hughes Medical Institute, USA) (Arriza *et al.*, 1987), while the plasmids expressing human ERα (pSG5-hERα) and ERβ (pSG5-hERβ) were received from Prof. F. Gannon (European Molecular

Biology Laboratory, Germany) (Flouriot *et al.*, 2000). The empty pSG5-expression vector was a gift from Prof. G. Mellgren (University of Bergen, Norway).

2.4   Plasmid DNA preparation

Competent *Escherichia coli* (*E. coli*) DH5α cells were transformed with the respective expression vectors using the heat shock method as previously described by Cohen *et al.* (1972). Transformed *E. coli* cells were grown on Luria-Bertani (LB) agar (Sigma-Aldrich, South Africa) plates (Addendum A) containing 0.1% (w/v) ampicillin (Sigma-Aldrich, South Africa), as all the plasmids used in this study contained an ampicillin resistance gene. A starter culture was prepared by inoculating a single transformed colony in 25 mL LB medium (Sigma-Aldrich, South Africa) (Addendum A) containing 0.1% (w/v) ampicillin and growing the culture at 37˚C for 6 hours at 150 revolutions per minute (rpm). An overnight culture was then prepared by transferring 250 µL of the starter culture to 250 mL LB medium containing 0.1% (w/v) ampicillin, and was grown at 37˚C for 16 hours at 150 rpm. In order to collect the bacterial cells, the cultures were centrifuged at 6000 x g for 10 minutes at 4˚C. Following centrifugation, the supernatant was discarded and the plasmid DNA was isolated using the Macherey-Nagel Nucleobond® Xtra Maxi kit (Separations Scientific, South Africa) according to the manufacturer's instructions. Cell pellets were resuspended in 12 mL resuspension buffer containing RNase A, and the cells were subsequently lysed by adding 12 mL lysis buffer and inverting the solution 5 times. The cell suspension was then incubated for 5 minutes at room temperature. Following incubation, 12 mL neutralization buffer was added and the solution inverted 15 times. The plasmid DNA was then purified using the Nucleobond® Xtra Maxi column with a filter insert, which was first equilibrated by adding 25 mL equilibration buffer. The cell lysate was then applied to the column filter. Once the cell debris was removed by gravitational flow, 15 mL of the equilibration buffer was allowed to flow through the column filter by gravitational flow. The filter was then discarded and the column washed with 25 mL wash buffer. The plasmid DNA was eluted by adding 15 mL elution buffer to the column. The eluate was collected in a 50 mL centrifuge tube to which 10.5 mL isopropanol (Sigma-Aldrich, South Africa) was added. The plasmid DNA was precipitated by centrifugation at 15 000 x g for 30 minutes at 4˚C. The supernatant was discarded and the pellet washed with 5 mL 70% (v/v) EtOH, followed by centrifugation at 15 000 x g for 5 minutes at room temperature. The DNA pellet was allowed to dry at room temperature before resuspension in 1 mL nuclease-free water. The concentration and purity of the isolated plasmid DNA were determined using the NanoDrop 1000 (Inqaba Biotech, South Africa), while the size and integrity of the plasmid DNA was analyzed by restriction enzyme digest and agarose gel electrophoresis.

2.5   Total RNA isolation

The human MCF-7 BUS, T47D and MDA-MB-453 breast cancer cell lines were maintained as described in Section 2.2. Cells were seeded into 12-well cell culture plates (Whitehead Scientific, South Africa) at a cell density of 1×10^5 cells per well in phenol red-free DMEM (Sigma-Aldrich, South Africa) supplemented with PenStrep (Sigma-Aldrich, South Africa), and either 5% (v/v) heat-inactivated charcoal-stripped (CS)-FCS (Addendum A) (MCF-7 BUS), 10% (v/v) CS-FCS (T47D) or 10% (v/v) CS-FCS plus 2 mM L-glutamine (MDA-MB-453). Cells were allowed to settle for 24 hours before treatment with unsupplemented phenol red-free DMEM containing either 0.1% (v/v) EtOH or 10 nM of the androgens and progestogens, in the absence and presence of 10 µM of the AR antagonist, OHF. Following incubation for 24 hours, the cells were washed with 1X phosphate-buffered saline (PBS) (Sigma-Aldrich, South Africa) before lysis by adding 400 µL Tri-Reagent® (Sigma-Aldrich, South Africa) to each well. Lysates were then transferred to 1.5 mL microcentrifuge tubes and incubated for 5 minutes at room temperature. This was followed by the addition of 80 µL chloroform, vortexing of samples for 15 seconds, and incubation of samples at room temperature for 3 minutes. Samples were subsequently centrifuged at 14 000 rpm for 20 minutes at 4°C for separation into three distinct phases: a clear aqueous phase containing RNA, an interphase containing DNA, and a pink organic phase containing protein. The RNA-containing aqueous phase was transferred to a clean 1.5 mL microcentrifuge tube and an equal volume of ice-cold isopropanol was added. Samples were vortexed for 5 seconds, incubated for 15 minutes at room temperature and centrifuged at 14 000 rpm for 15 minutes at 4°C. The supernatant was discarded and the RNA pellets were washed with 500 µL 75% (v/v) EtOH in diethyl pyrocarbonate (DEPC)-treated water. Samples were vortexed for 1 minute and centrifuged at 8 000 rpm for 10 minutes at 4°C. Thereafter, the supernatant was discarded and the RNA pellets were allowed to air dry on ice. The RNA pellets were subsequently dissolved in 15 µL DEPC-treated water. The concentration and purity of each sample was measured using the NanoDrop 1000 (Inqaba Biotech, South Africa), while the integrity of the RNA was confirmed by assessing the presence of intact 18S and 28S ribosomal RNA subunits using denaturing agarose gel electrophoresis (Figure C1, Addendum C). All RNA samples were subsequently stored at -80°C.

2.6   cDNA synthesis

Following the total RNA isolation, cDNA was synthesized by reverse transcription of the RNA using the Promega ImProm-II™ reverse transcription system (Anatech, South Africa) as per the manufacturer's instructions. Briefly, 0.5 µg total RNA, a final concentration of 30 ng/µL oligo(dT)$_{15}$ primer and PCR-grade water (where needed) was added to a final volume of 2.5 µL in a thin-walled

PCR tube. To denature the RNA secondary structures, the template-primer mixture was incubated for 5 minutes at 70°C and subsequently placed on ice for 5 minutes. A master mix containing the rest of the components needed for the reverse transcription process was prepared by adding 3.5 µL PCR-grade water, 2 µL Im-Prom-II™ 5X reaction buffer, 0.75 µL MgCl$_2$ (final concentration of 1.5 mM), 0.5 µL deoxynucleotide triphosphate (dNTP) mix (final concentration of 0.5 nM for each nucleotide), 0.25 µL recombinant RNasin ribonuclease inhibitor (10 U per reaction), and 0.5 µL ImProm-II reverse transcriptase (80 U per reaction), per RNA sample. A volume of 7.5 µL of the master mix was added to each tube containing the denatured RNA and samples were incubated for 5 minutes at 25°C to allow for annealing of the primers, followed by incubation for 1 hour at 45°C for extension. Thereafter, samples were incubated for an additional 15 minutes at 70°C to inactivate the Im-Prom-II™ reverse transcriptase enzyme. All cDNA samples were stored at -20°C until analysis.

2.7 Real-time quantitative polymerase chain reaction (qPCR)

The relative mRNA expression levels of ERα, ERβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by real-time qPCR using the primers described in Table 2.2, the KAPA SYBR FAST® qPCR master mix (Roche Applied Science, South Africa), and the LightCycler 96 system (Roche Applied Science, South Africa) according to the manufacturer's instructions. The reaction mixture per well of the PCR plate was as follows: 1 µL cDNA or PCR-grade water (negative control), forward and reverse primers (final concentration of 0.5 µM each), 5 µL KAPA SYBR FAST® qPCR master mix, and PCR-grade water to a final volume of 10 µL. PCR plates containing the samples were pre-incubated in the LightCycler 96 system for 5 minutes at 95°C in order to activate the Taq polymerase. Details of the three subsequent PCR amplification steps are described in Table 2.2. Quantification cycle (C$_q$) values were used to calculate the relative transcript levels of ERα, ERβ and GAPDH using the mathematical method previously described by Pfaffl (2001). GAPDH was used as an internal control. Further details on the real-time qPCR method, such as primer efficiencies and melting curve analysis, are discussed in Addendum C.

Table 2.2. Primer sequences and thermal cycling conditions for genes investigated in this study.

Gene	Primers (5' – 3')	Strand	Amplicon length (bp)	Thermal cycling profile				Reference
				Denaturation	Annealing	Elongation	Number of cycles	
ERα	AGATCTTCGACATGCTGCTGGCTA	Forward	137	95°C for 3 s	63°C for 20 s	72°C for 10 s	43	Qiao *et al.* (2012)
	AGACTTCAGGGTGCTGGACAGAAA	Reverse						
ERβ	TAGTGGTCCATCGCCAGTTAT	Forward	393	95°C for 30 s	56°C for 15 s	72°C for 60 s	40	Enmark *et al.* (1997)
	GGGAGCCACACTTCACCAT	Reverse						
GAPDH	TGAACGGGAAGCTCACTGG	Forward	307	95°C for 10 s	59°C for 10 s	72°C for 12 s	35	Ishibashi *et al.* (2003)
	TCCACCACCCTGTTGCTGTA	Reverse						

2.8   Western blot

For the characterization of the MCF-7 BUS, T47D and MDA-MB-453 human breast cancer cell lines in terms of their steroid receptor content, cells were maintained as described in Section 2.2, and seeded into 12-well cell culture plates at a density of 1 x 10^5 cells per well. For positive controls, COS-1 cells were transiently transfected with 250 ng of the expression vectors for the GR, PR-A or PR-B isoforms, while HEK-293 cells were transiently transfected with expression vectors for the AR, ERα or ERβ, using XtremeGENE HP DNA transfection reagent (Roche Applied Science, South Africa) as per the manufacturer's instructions. Untransfected COS-1 and HEK-293 cells were used as negative controls. All cells were grown until 100% confluency and washed with 1X PBS before cell lysis in 80 μL 2X Laemmli buffer (Addendum A). Cell lysates were denatured by boiling for 10 minutes at 97°C, and samples stored at -20°C until analysis.

For investigation into the effects of the test compounds on AR, ERα and ERβ protein expression, MCF-7 BUS or MDA-MB-453 cells were seeded into 12-well cell culture plates at a density of 1 x 10^5 cells per well and treated for 24 hours with unsupplemented phenol red-free DMEM containing either 0.1% (v/v) EtOH or 10 nM of the test compounds in the absence and presence of 10 μM of the AR antagonist, OHF. Following treatment, cell lysates were prepared and denatured as described above.

Proteins were separated on a 10% SDS-polyacrylamide gel in 1X SDS-PAGE Running Buffer (Addendum A) at 100 V for 15 minutes and then at 200 V for 30 - 45 minutes. The PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, South Africa) was loaded onto each SDS-polyacrylamide gel in order to determine protein sizes. Following electrophoresis, proteins were electroblotted onto nitrocellulose membranes (AEC-Amersham Biosciences, South Africa) at 180 mA for 2 hours in ice-cold 1X Transfer Buffer (Addendum A) using a mini Trans-Blot system (Bio-Rad, South Africa). Membranes were subsequently blocked for 90 minutes in 10% (w/v) fat-free milk powder prepared in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (Sigma Aldrich, South Africa) (TBST) (Addendum A). Thereafter, the membranes were rinsed with TBST and subsequently probed for 16 hours at 4°C with the appropriate primary antibody at the dilutions specified in Table 2.3. GAPDH was used as the loading control. Membranes were then washed once for 15 minutes and thrice for 5 minutes using TBST. Following the wash steps, the membranes were incubated with either goat anti-rabbit or mouse IgG kappa binding protein (IgGκ BP) horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.3) for 90 minutes at room temperature. The membranes were subsequently washed as above, and proteins visualized using the Clarity™ Western ECL Substrate (Bio-Rad, South Africa) and the MyECL imaging system (Thermo Fisher

Scientific, South Africa) according to the manufacturer's instructions. The MyImageAnalysis™ software (Thermo Fisher Scientific, South Africa) was used to analyze and quantify protein expression.

Table 2.3. Primary and secondary antibody dilutions used for western blotting.

Protein	Primary antibody	Dilution	HRP-conjugated secondary antibody	Dilution
AR	AR (441)	1:1000	Mouse IgGκ BP	1:3000
ERα	ERα (F-10)	1:1000	Mouse IgGκ BP	1:1000
ERβ	ERβ (EPR3777)	1:500	Goat anti-rabbit	1:1000
	ERβ (EPR20743)	1:1000		
GR	GR (H-300)	1:2000	Goat anti-rabbit	1:3000
PR-A/B	PR (NCL-L-PGR-312)	1:1000	Mouse IgGκ BP	1:2000
GAPDH	GAPDH (0411)	1:3000	Mouse IgGκ BP	1:3000

All antibodies, except the ERβ and PR-A/B antibodies, were obtained from Santa Cruz Biotechnology, Inc., Europe. The ERβ antibodies were purchased from Abcam, USA, while the PR-A/B primary antibody was purchased from Leica Biosystems Inc., USA.

2.9   Mammalian two-hybrid assay

Mammalian two-hybrid assays, illustrated in Figure 2.1, were performed as previously described by Africander *et al*. (2014) with minor modifications. Briefly, COS-1 cells were seeded into 10 cm cell culture dishes at a density of 2 x 10^6 cells in phenol red-free DMEM supplemented with 10% (v/v) CS-FCS and PenStrep. The next day, cells were transiently transfected with 6.67 µg of the pTAT-GRE-E1b-luc promoter-reporter construct (referred to as the classical ARE), 0.67 µg of pSG5-hAR (DBD-LBD) and 0.67 µg of pSNATCH-II-hAR (NTD), using XtremeGENE HP DNA transfection reagent (Roche Applied Science, South Africa) according to the manufacturer's instructions. Following a 24-hour incubation period, cells were trypsinized and re-seeded into 96-well cell culture plates at a density of 1 x 10^4 cells per well and allowed to settle. Cells were then treated for 24 hours with unsupplemented phenol red-free DMEM containing either 0.2% (v/v) EtOH or (i) 100 nM DHT or the progestogens (agonist mode) or (ii) 100 nM DHT in the absence and presence of increasing concentrations of the progestogens (antagonist mode). Following treatment, cells were washed with 1X PBS and lysed using 25 µL passive lysis buffer (Addendum A). The luciferase activity in the cell lysates was measured using the Luciferase Assay System (Promega, USA) and a Veritas microplate luminometer (Whitehead Scientific, South Africa). The values obtained were expressed in relative

light units (RLU) and normalized to the total protein concentration (mg/mL), which was determined using the Bradford protein determination method (Bradford 1976).
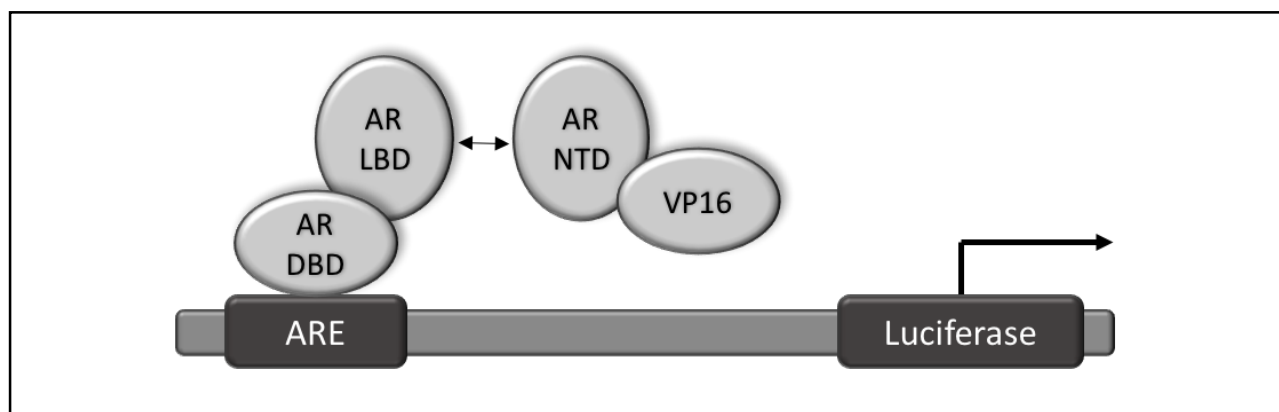

Figure 2.1. Principle of the mammalian two-hybrid assay used to study the AR N/C interaction. The interaction between the N-terminal domain (NTD) and carboxyl-terminal of the AR (so-called AR N/C interaction) was studied using the *in vitro* mammalian two-hybrid assay. Vectors encoding the carboxyl-terminal AR ligand binding domain (LBD) fused to the DNA binding domain (DBD) of the AR (pSG5-hAR (DBD-LBD)), as well as the NTD of the AR fused to the activation domain of the herpes simplex virus protein, VP16 (pSNATCH-II-hAR (NTD)), were co-transfected into the COS-1 cell line together with a classical androgen response element (ARE)-driven luciferase reporter construct. Cells were treated with the test compounds, and their ability to induce the AR N/C interaction or antagonize the DHT-induced AR N/C interaction was determined by measuring luciferase activity using a luminometer. Figure adapted from Africander *et al.* (2014).

2.10   Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was used to measure breast cancer cell proliferation. The human T47D and MCF-7 BUS breast cancer cell lines were maintained as described in Section 2.2 and seeded into 96-well cell culture plates at 5×10^3 cells per well in phenol red-free DMEM supplemented with PenStrep, and either 10% (v/v) CS-FCS (T47D) or 5% (v/v) heat-inactivated CS-FCS (MCF-7 BUS). The MDA-MB-453 breast cancer cell line was maintained as described in Section 2.2, and plated in 6 cm cell culture dishes at a density of 8×10^5 cells in phenol-red DMEM supplemented with PenStrep, 10% (v/v) FCS and 2 mM L-glutamine. The next day, MDA-MB-453 cells were transiently transfected with 300 ng of the expression vectors for (i) ERα or (ii) ERα and ERβ using XtremeGENE HP DNA transfection reagent (Roche Applied Science, South Africa) according to the manufacturer's instructions. Following a 24-hour incubation period, the MDA-MB-453 cells were trypsinized and re-seeded into 96-well cell culture plates at a density of 5×10^3 cells per well in phenol red-free DMEM supplemented with PenStrep, 10% (v/v) FCS and 2 mM L-glutamine. The next day, the MDA-MB-453 cells were treated

with either 0.1% (v/v) EtOH or increasing concentrations of E_2, in the absence and presence of equimolar concentrations of DHT. Twenty-four hours after the T47D and MCF-7 BUS cells were seeded, these cells were treated with either 0.2% (v/v) EtOH or 10 nM Mib, DHT, or the progestogens, in the absence and presence of either 1 or 10 µM steroid receptor-selective antagonist. Following a 72-hour incubation period, all cells were re-treated with the test compounds for an additional 44 hours, before incubation with pre-warmed 5 mg/mL MTT (Addendum A) solution for 4 hours. The medium was carefully aspirated and 200 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, South Africa) was added to each well in order to solubilize the formazan crystals. Absorbances were then measured at 550 nm using the BioTek® Power Wave 340 microplate spectrophotometer (Analytical and Diagnostic Products, South Africa). Results were represented as fold induction relative to the vehicle control (0.1 or 0.2% EtOH), which was set as one.

2.11   Data manipulation and statistical analysis

GraphPad Prism® version 7.00 (GraphPad Software, USA) was used for statistical analysis and graphical representation. Statistical significance of ungrouped data was determined using one-way analysis of variance (ANOVA) with either Dunnett's (compares all columns vs. control column), Bonferroni's (compares all columns to each other) or Newman Keuls (compares all columns to each other) as post-test. Statistical analyses on data with two or more data sets (grouped data) were performed using two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test, unless otherwise indicated. Statistical significance is denoted by either *, **, or ***, to indicate $p<0.05$, $p<0.01$ or $p<0.001$, respectively, while no statistical significance ($p>0.05$) is denoted by ns. Alphabetical letters are also used to indicate statistical significance, where values that are statistically different are assigned different letters. Unless otherwise indicated, the error bars represent the standard error of the mean (SEM) of three independent experiments.

Chapter 3

Results

3.1   Progestins elicit different AR conformations.

A number of parameters contribute to the biological activity of a ligand via a steroid receptor, and include the affinity of the ligand for the receptor, the rate of its nuclear translocation, the induced receptor conformation and recruitment of co-regulators (Griekspoor *et al.*, 2007). In terms of the AR, the interaction between the amino- and carboxyl-terminal of the AR, commonly known as the AR N/C interaction has been well described for AR agonists (He *et al.*, 1999, 2000; Kemppainen *et al.*, 1999; Schaufele *et al.*, 2005; Van Royen *et al.*, 2007, 2012; Africander *et al.*, 2014). Interestingly, it has previously been shown that while both the first generation progestins, MPA and NET-A, display androgenic properties similar to each other and the natural androgen DHT, only DHT and NET-A could induce the AR N/C interaction (Africander *et al.*, 2014). MPA on the other hand, has been shown to be an antagonist of the DHT-induced AR N/C interaction (Kemppainen *et al.*, 1999; Tanner, 2002). It has recently been shown that LNG and GES also display androgenic activity similar to DHT, while NES, NOMAC and DRSP display anti-androgenic activity similar to the natural progestogen, P_4 (Louw-du Toit *et al*. 2017a). Whether these progestins induce or antagonize the AR N/C interaction is not known. A mammalian two-hybrid assay was thus used to compare the potential of LNG, GES, NES, NOMAC and DRSP to induce the AR N/C interaction. COS-1 cells were transiently transfected with a promoter reporter construct, the expression vector encoding the human AR DBD-LBD and the expression vector encoding the human AR NTD. Results in Figure 3.1A show that LNG and GES induce the AR N/C interaction to the same extent as each other and NET-A, but weaker than DHT. In contrast, NES, NOMAC, and DRSP did not induce the AR N/C interaction (Figure 3.1A). The result showing that P_4 and MPA do not induce the AR N/C interaction, while NET-A does, is consistent with the previous study from our group (Africander *et al.*, 2014). None of the progestins, except MPA, inhibited the DHT-induced AR N/C interaction (Figure 3.1B).

3.2   Unlike the androgens Mib and DHT, the progestogens do not influence AR protein levels in the MCF-7 BUS breast cancer cell line.

It is known that the binding of androgens to the AR stabilizes the AR protein, while the binding of anti-androgens result in AR protein degradation (Kemppainen *et al.*, 1992; Zhou *et al.*, 1995). We thus evaluated whether progestins with androgenic activity similar to DHT would stabilize AR protein in a similar manner as DHT, while progestins with anti-androgenic activity similar to P_4, would cause AR degradation. Western blot analysis was performed with lysates from the MCF-7 BUS breast cancer cell line treated with the androgens, Mib and DHT, the androgenic progestins, MPA, NET-A and LNG, or the anti-androgenic progestogens, P_4, NES and NOMAC, in the absence and presence of the AR antagonist, OHF. The results showed that both Mib and DHT increase AR

protein levels (Figure 3.2B), indicating stabilization of the AR protein. The Mib- and DHT-induced increase was inhibited in the presence of the AR antagonist, OHF (Figure 3.2C). Knowing that androgens stabilize the AR protein, while it is degraded by anti-androgens, it was surprising that neither the androgenic nor anti-androgenic progestogens affected AR protein levels in the MCF-7 BUS cell line (Figure 3.2B).
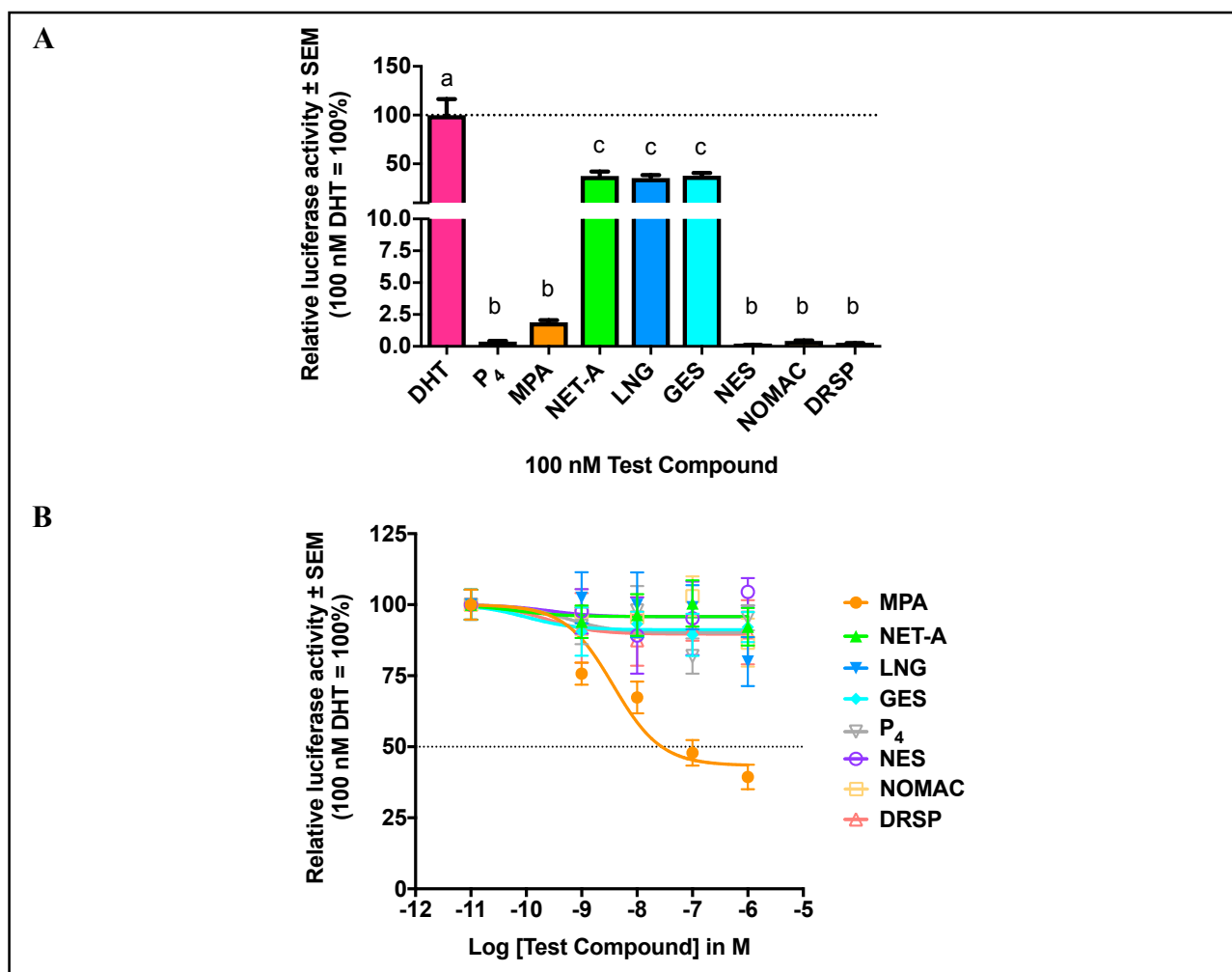

Figure 3.1. (A) LNG and GES, induce the AR N/C interaction, while NES, NOMAC and DRSP do not. (B) Only MPA antagonizes the DHT-induced AR N/C interaction and in a dose-dependent manner. COS-1 cells were transiently transfected with the pTAT-GRE-E1b-luc promoter-reporter construct, as well as the pSNATCH-II-hAR (NTD) and wild-type pSG5-hAR (DBD-LBD) expression vectors. Cells were incubated for 24 hours with either 0.2% (v/v) EtOH (vehicle), (A) 100 nM DHT, P_4, MPA, NET-A, LNG, GES, NES, NOMAC or DRSP, or (B) 100 nM DHT, in the absence and presence of increasing concentrations of P_4, MPA, NET-A, LNG, GES, NES, NOMAC or DRSP. Luciferase activity was measured in relative light units and normalized to the protein concentration determined using the Bradford method (Bradford, 1976). Induction is shown as % luciferase activity relative to DHT = 100%. The result shown is the average of at least three independent experiments with each condition performed in triplicate (± SEM). Statistical analysis was performed using one-way ANOVA with Bonferroni's (compares all columns to each other) as post-test.
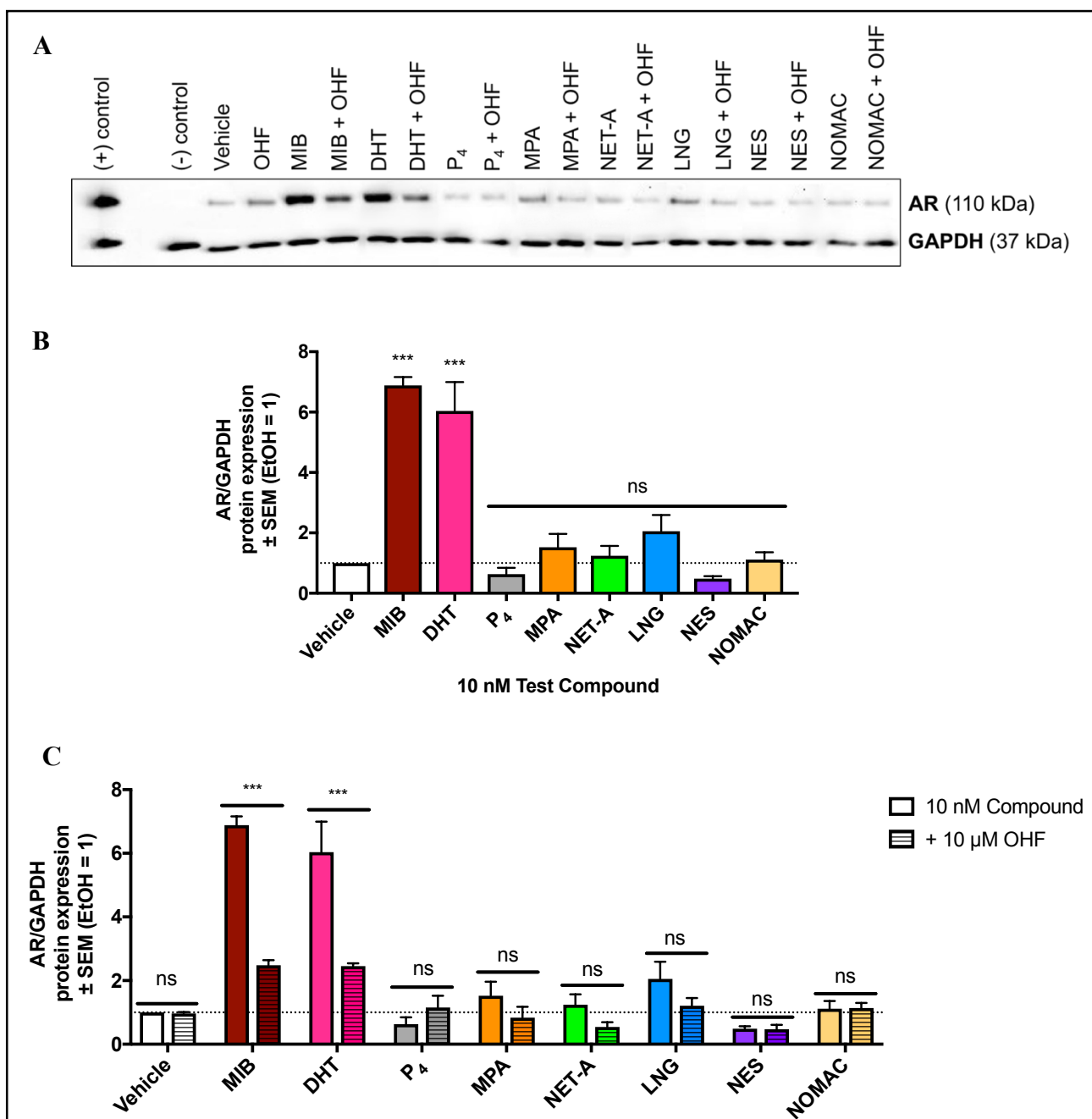
Figure 3.2. Progestogens, unlike Mib and DHT, do not influence AR protein levels in MCF-7 BUS cells.
The human MCF-7 BUS breast cancer cells were treated for 24 hours with either 0.2% (v/v) EtOH (vehicle) or 10 nM Mib, DHT, P_4, MPA, NET-A, LNG, NES or NOMAC, in the absence and presence of 10 μM OHF. Protein lysates were analyzed by western blotting using primary antibodies specific for the AR and GAPDH (loading control). HEK-293 cells transiently transfected with an expression vector for the AR was used as a positive control, while untransfected COS-1 cells were used as a negative control. (A) A representative western blot of at least three independent experiments is shown. (B, C) AR and GAPDH protein expression was quantified using the MyImageAnalysis™ software, and AR expression was normalized to GAPDH expression. The results shown are the averages (± SEM) of at least three independent experiments. Statistical analysis was performed using (B) one-way ANOVA with Dunnett's (compares all columns vs. control column) as post-test or (C) two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

3.3   All progestogens increase proliferation of the human MCF-7 BUS and T47D breast cancer cell lines to the same extent.

Results from *in vitro* studies investigating the effects of androgens and progestogens on breast cancer cell proliferation are conflicting, with some studies showing increased proliferation (Birrell *et al.* 1995b; Schoonen *et al.* 1995a; Schoonen *et al.* 1995b), while others show a decrease (Botella *et al.*, 1994; Formby and Wiley, 1998; Peters *et al.*, 2009), or no effect (Van Der Burg *et al.*, 1992; Catherino *et al.*, 1993; Ruan *et al.*, 2012). We thus directly compared the effects of the selected progestins relative to each other, P_4, the synthetic androgen, Mib, and natural androgen, DHT, on the proliferation of the MCF-7 BUS and T47D breast cancer cell lines. Both these cell lines are widely used in breast cancer research (Aka and Lin, 2012). The results show that all progestins increase breast cancer cell proliferation to the same extent as each other and DHT in T47D cells (Figure 3.3B). Similar results were observed in the MCF-7 BUS cells, except that LNG does not the increase cell proliferation to the same extent as DHT (Figure 3.3A).
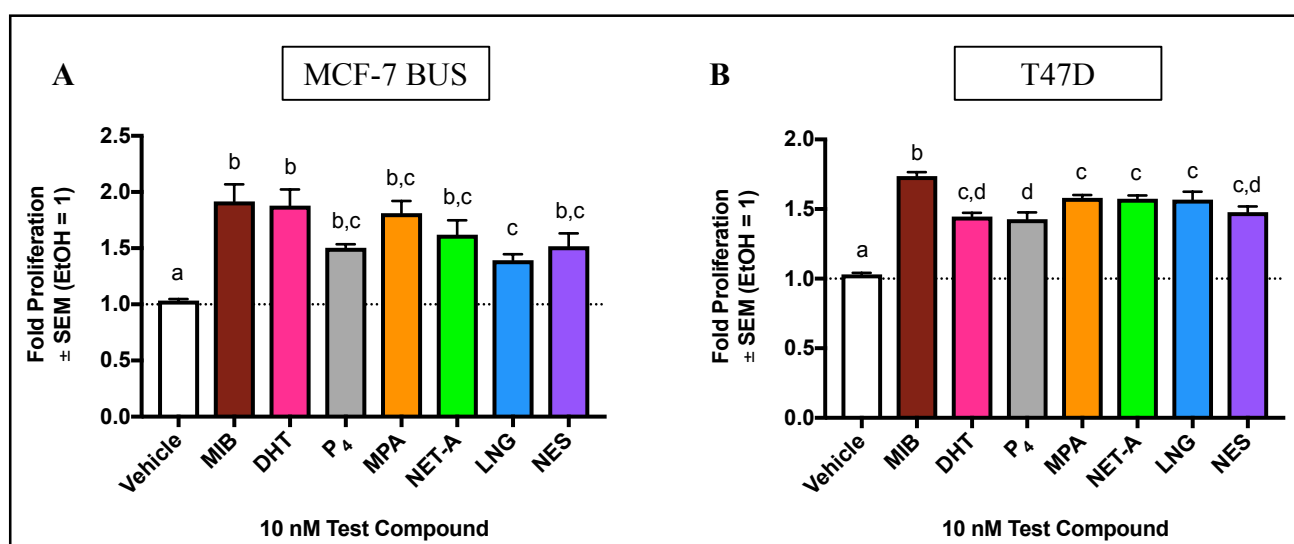

Figure 3.3. All progestogens increase human breast cancer cell proliferation. The human (A) MCF-7 BUS and (B) T47D breast cancer cell lines were treated with either 0.1% (v/v) EtOH (vehicle) or 10 nM Mib, DHT, P_4, MPA, NET-A, LNG or NES for 72 hours. The medium was aspirated and cells were re-treated for an additional 44 hours. Cell proliferation was measured using the MTT cell viability assay. Results are represented as fold proliferation relative to the vehicle control set as one. Results shown are the averages (± SEM) of at least three independent experiments with each condition performed in triplicate. Statistical analysis was performed using one-way ANOVA with Newman-Keuls (compares all columns to each other) as post-test.

3.4   While the AR mediates the proliferative effects of MPA, NET-A and LNG in the MCF-7 BUS breast cancer cells, multiple receptors are required for their proliferative effects in the T47D breast cancer cells.

Having shown that progestins known to display androgenic properties, with the exception of LNG in the MCF-7 BUS cells, increase breast cancer cell proliferation to the same extent as DHT, we postulated that the AR may be mediating these effects. However, as some progestins bind to multiple steroid receptors, we also investigated the role of the PR, GR and ER. First, we confirmed previous reports that the MCF-7 BUS and T47D human breast cancer cell lines express all of the above-mentioned steroid receptors (Horwitz *et al.*, 1975, 1978; Vladusic *et al.*, 2000; Singer *et al.*, 2003). Western blot analysis of the cell lysates showed that both the MCF-7 BUS and the T47D cell lines express PR-A, PR-B, the AR, GR, ERα and ERβ (Figure 3.4).
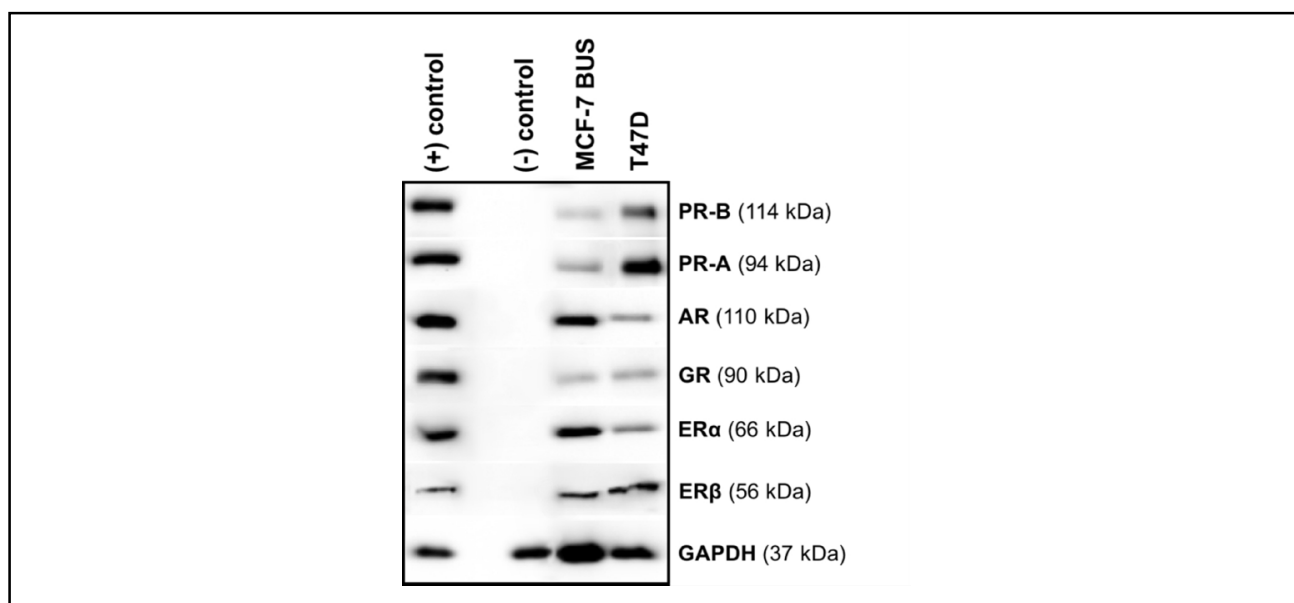

Figure 3.4. MCF-7 BUS and T47D breast cancer cell lines both express endogenous PR-A, PR-B, AR, GR, ERα and ERβ. Whole cell extracts were prepared from MCF-7 BUS and T47D cells and analyzed by western blotting using primary antibodies specific for PR-A/B, AR, GR, ERα, ERβ and GAPDH (loading control). COS-1 cells transiently transfected with expression vectors for either PR-A, PR-B or GR, or HEK-293 cells transiently transfected with expression vectors for either the AR, ERα or ERβ, were used as positive controls. Untransfected COS-1 or HEK-293 cells were used as negative controls. A representative western blot of at least three independent experiments is shown.

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Next, the cells were treated with the androgens and progestogens in the absence and presence of specific steroid receptor antagonists. As expected, the results in Figure 3.5 show that the addition of the AR antagonist, OHF, inhibited the effects of Mib and DHT, confirming a previous report that these effects are mediated via the AR (Birrell *et al*. 1995b). OHF also significantly inhibited the effects of MPA, NET-A and LNG in the MCF-7 BUS cells (Figure 3.5A), suggesting that these effects are also mediated via the AR. We did not see any significant change in progestin-induced proliferation of the MCF-7 BUS cells in the presence of RU486, a well-known PR and GR antagonist (Spitz & Bardin 1993) (Figure 3.5C). Surprisingly, OHF only partially inhibited the effects of MPA, NET-A and LNG on T47D proliferation (Figure 3.5B), suggesting that the AR is not the only steroid receptor involved in mediating their effects in this cell line. The results in Figure 3.5D show that RU486 decreases progestogen-induced proliferation of the T47D cells. Considering that we show that RU486 acts as a PR-, but not a GR-, antagonist in this cell line (Figure B2, Addendum B), this result suggests that the PR is required for the effects of the progestogens on T47D cell proliferation. Interestingly, RU486 itself was shown to increase proliferation of both MCF-7 BUS (Figure 3.5C) and T47D (Figure 3.5D) breast cancer cells, which is consistent with previous studies showing that RU486 elicits agonist activity in both these cell lines (Catherino *et al.*, 1993; Kalkhoven *et al.*, 1994). Although the results in Figure 3.5E show a decrease in MCF-7 BUS cell proliferation with all ligands in the presence of the ER antagonist, ICI, this was likely due to a decrease in cell viability induced by ICI (Figure B3, Addendum B). We could thus not draw any conclusions about the role of the ER in the proliferation of MCF-7 BUS cells. In contrast, ICI did not affect the viability of the T47D cells. The results in Figure 3.5F show that NET-A-induced proliferation of T47D cells was inhibited by ICI, while ICI partially inhibited LNG-induced proliferation. As neither NES nor P_4 bind to the ER, nor display anti-androgenic properties, it was not surprising that they did not increase cell proliferation via the ER or AR in either cell line.
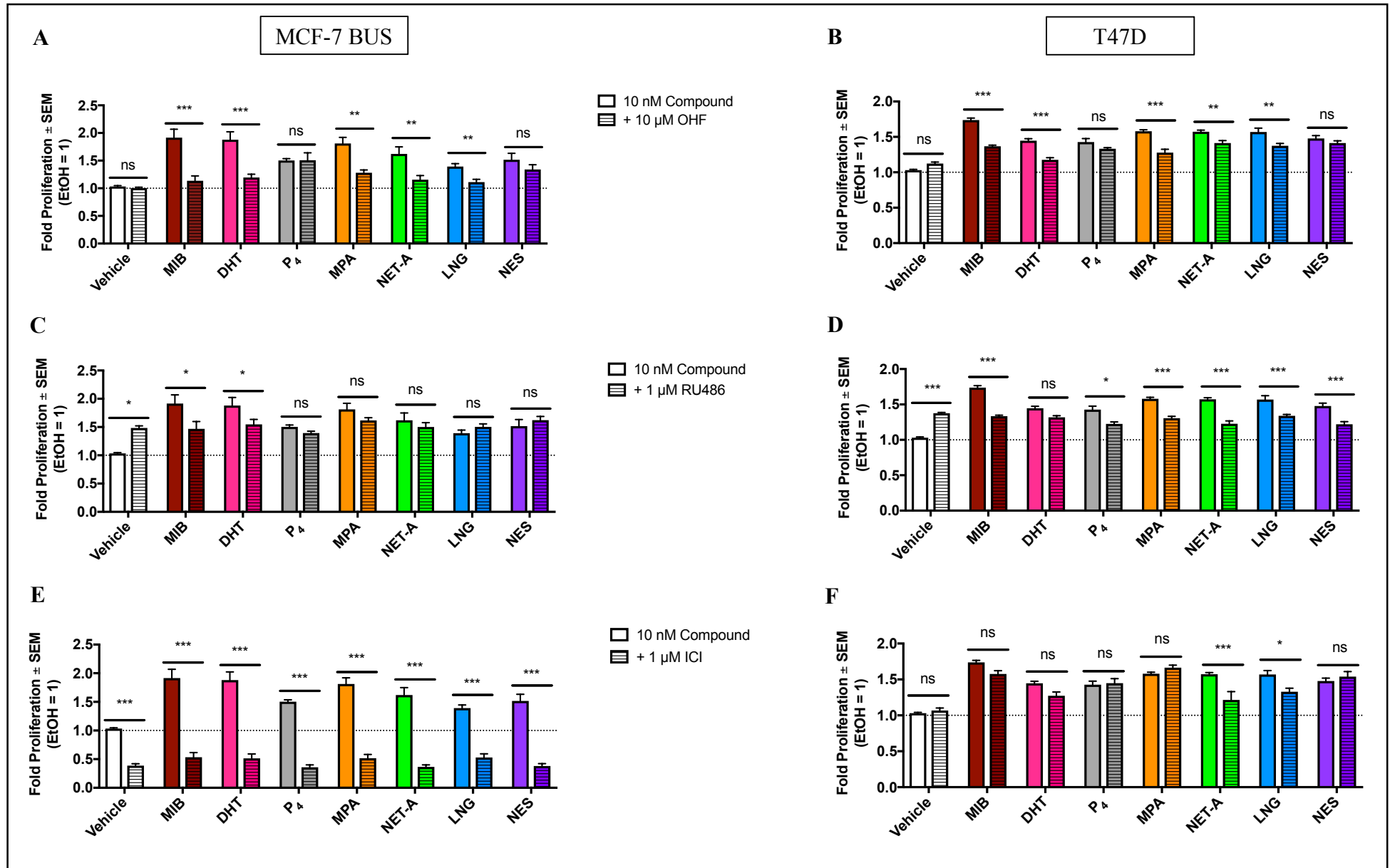
Figure 3.5 on next page.

Figure 3.5. While only the AR mediates the proliferative effects of MPA, NET-A and LNG in the MCF-7 BUS cells, multiple receptors are required for their proliferative effects in the T47D cells. The human (A, C, E) MCF-7 BUS and (B, D, F) T47D breast cancer cells were treated with either 0.2% (v/v) EtOH (vehicle) or 10 nM Mib, DHT, P$_4$, MPA, NET-A, LNG or NES, in the absence and presence of (A-B) 10 μM OHF, (C-D) 1 μM RU486 or (E-F) 1 μM ICI, for 72 hours. The medium was aspirated and cells were re-treated for an additional 44 hours. Cell proliferation was measured using the MTT cell viability assay. Results are represented as fold proliferation relative to the vehicle control. The results shown are the averages (± SEM) of at least three independent experiments with each condition performed in triplicate. Statistical analysis was performed using two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

3.5   Androgens increase ERβ mRNA expression in both MCF-7 BUS and T47D breast cancer cells.

It has recently been shown that the synthetic androgen, Mib, and the natural androgen, DHT, increase ERβ expression to the same extent as each other in MCF-7 breast cancer cells, via a mechanism requiring the AR (Rizza *et al.*, 2014). We thus validated these findings in the MCF-7 BUS cell line, which is a clone of the MCF-7 breast cancer cell line that is more estrogen-sensitive (Villalobos *et al.*, 1995). Real-time qPCR results in Figure 3.6A confirm that both Mib and DHT increase ERβ mRNA expression in MCF-7 BUS breast cancer cells, albeit to different extents. To exclude the possibility of cell-specific effects, we repeated the experiment in T47D breast cancer cells and showed that Mib and DHT also upregulated ERβ mRNA expression to different extents in these cells (Figure 3.6B). However, DHT increased ERβ mRNA expression to a greater extent than Mib in the T47D cells, while the reverse is true in the MCF-7 BUS cells (Figure 3.6C). In order to confirm that the effects of the androgens were via the AR, MCF-7 BUS cells were treated with Mib and DHT, in the absence and presence of the AR antagonist, OHF. Surprisingly, although we had detected ERβ mRNA expression in two biological replicate experiments in both the MCF-7 BUS (Figure 3.6A) and T47D (Figure 3.6B) cells, we were unable to reproduce these results when performing the experiments with the antagonist. Thus, we could not establish the role of the AR in mediating the effects of Mib and DHT on ERβ mRNA expression. Investigations into effects on ERβ protein expression in these cell lines could thus also not be performed.
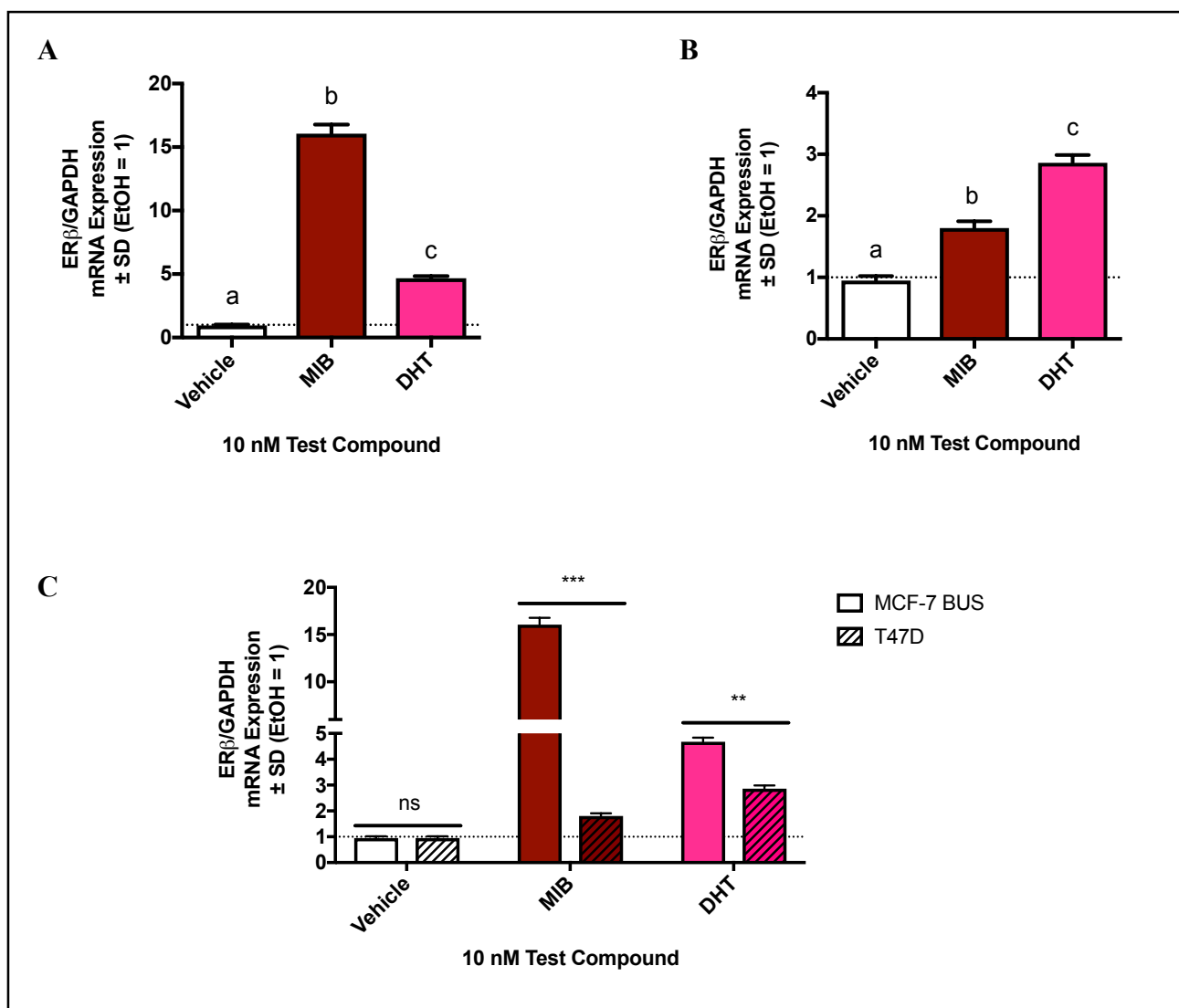
Figure 3.6. Mib and DHT differentially increase ERβ mRNA expression in MCF-7 BUS and T47D breast cancer cells. The human (A) MCF-7 BUS and (B) T47D breast cancer cells were treated with either 0.1% (v/v) EtOH (vehicle), 10 nM Mib or DHT for 24 hours. Total RNA was isolated, cDNA synthesized, and ERβ and GAPDH mRNA expression analyzed using real-time qPCR. Results shown are the averages (±SD) of two independent experiments. The ratio of ERβ to GAPDH mRNA expression of the treated samples was calculated relative to that of the vehicle control. (C) The data from (A) and (B) were re-plotted to directly compare the androgen-induced effects between the MCF-7 BUS and T47D cell lines. Statistical analysis was performed using (A, B) one-way ANOVA with Newman-Keuls (compares all columns to each other) as post-test or (C) two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

3.6   While Mib and DHT decrease ERα mRNA expression via the AR in MCF-7 BUS breast cancer cells, the NET-A-induced decrease in ERα expression does not require the AR.

Having confirmed that both Mib and DHT increase ERβ mRNA expression in both the MCF-7 BUS and T47D breast cancer cells, albeit to different extents, we next investigated whether these androgens would elicit similar effects on ERα expression in the MCF-7 BUS cell line. The cells were thus treated as described in Section 3.5, and real-time qPCR results show that both Mib and DHT significantly decreased ERα mRNA expression (Figure 3.7A). The addition of AR antagonist, OHF, reversed the effects of both Mib and DHT (Figure 3.7B), indicating that the Mib- and DHT-induced decrease in ERα mRNA expression is mediated by the AR. Western blot analysis of lysates from MCF-7 BUS cells treated as above, showed that Mib and DHT had no effect on ERα protein expression (Figure 3.7D).

Considering that Mib and DHT decreased ERα mRNA expression via the AR, we next investigated whether progestins with androgenic properties similar to DHT would elicit similar effects. Results show that NET-A downregulated both the mRNA (Figure 3.8A) and protein (Figure 3.8D) expression of ERα, while MPA and LNG had no effect. However, the addition of the AR antagonist, OHF, did not decrease the effects of NET-A on ERα mRNA (Figure 3.8B) or protein (Figure 3.8E) expression, suggesting that the AR is not involved. NES and NOMAC had no effect on ERα mRNA (Figure 3.8A) or protein (Figure 3.8D) expression. Results also show that P_4 decreased ERα mRNA expression (Figure 3.8A), while having no effect on ERα protein expression (Figure 3.8D). Given the anti-androgenic properties of P_4 (Africander *et al.*, 2014; Louw-du Toit *et al.*, 2017a), it is surprising that the results in the presence of OHF show that, like the androgens, P_4 decreased ERα mRNA expression via a mechanism requiring the AR (Figure 3.8B).
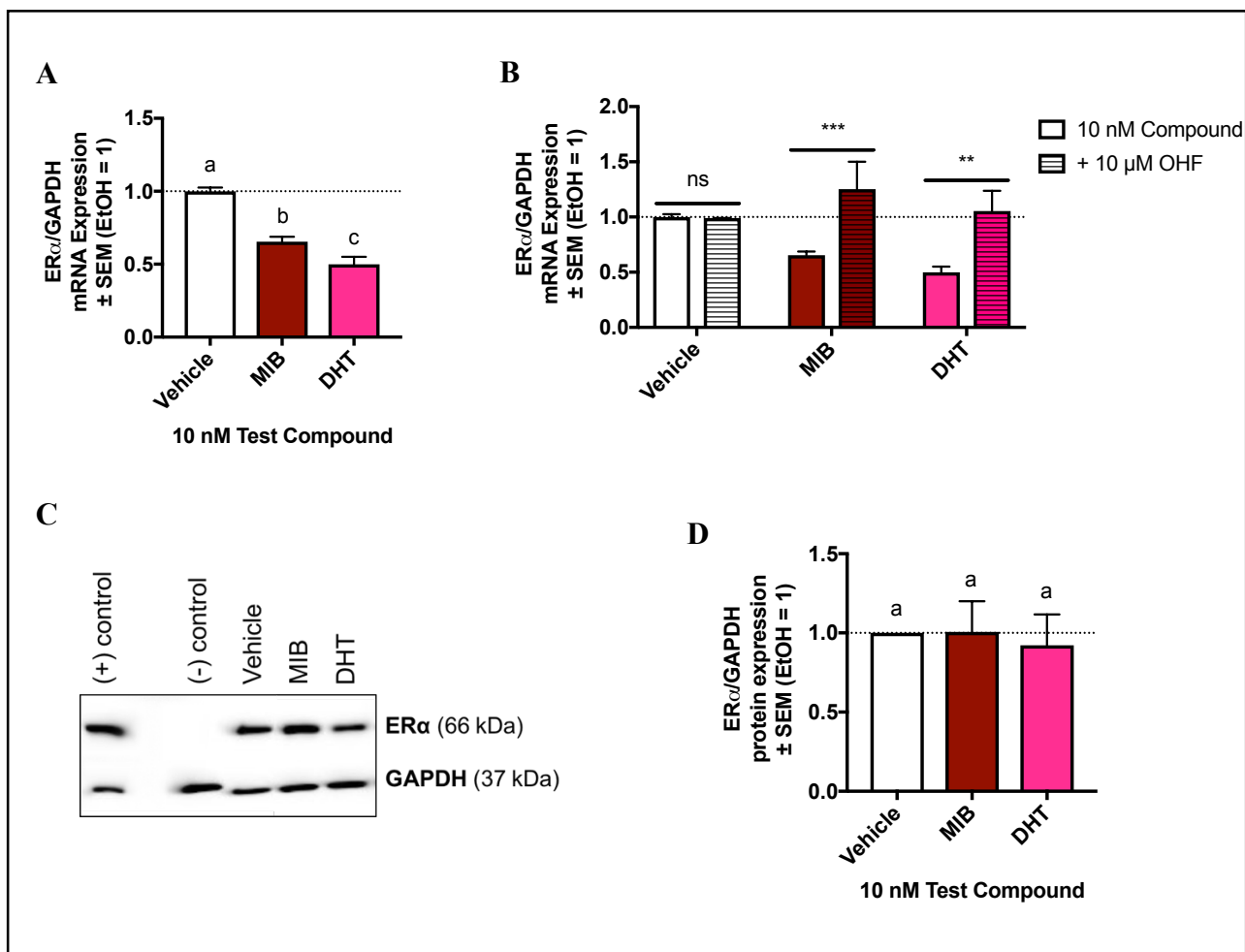
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Figure 3.7. Mib and DHT decreased ERα mRNA expression via an AR-mediated mechanism in MCF-7 BUS cells, while having no effect on ERα protein expression. The human MCF-7 BUS breast cancer cells were treated for 24 hours with either 0.2% (v/v) EtOH (vehicle), 10 nM Mib or DHT, in the absence and presence of 10 μM OHF. (A, B) Total RNA was isolated, cDNA synthesized, and ERα and GAPDH mRNA expression analyzed using real-time qPCR. The ratio of ERα to GAPDH mRNA expression of the treated samples was calculated relative to that of the vehicle control. (C, D) Protein lysates were analyzed by western blotting using primary antibodies specific for ERα and GAPDH (loading control). HEK-293 cells transiently transfected with an expression vector for ERα was used as a positive control, while untransfected COS-1 cells were used as a negative control. (C) A representative western blot is shown. (D) ERα protein expression was quantified using the MyImageAnalysis™ software, and normalized to GAPDH expression. Results shown for qPCR and western blotting are the averages (± SEM) of at least three independent experiments. Statistical analysis was performed using (A, D) one-way ANOVA with Newman-Keuls (compares all columns to each other) as post-test or (B) two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.
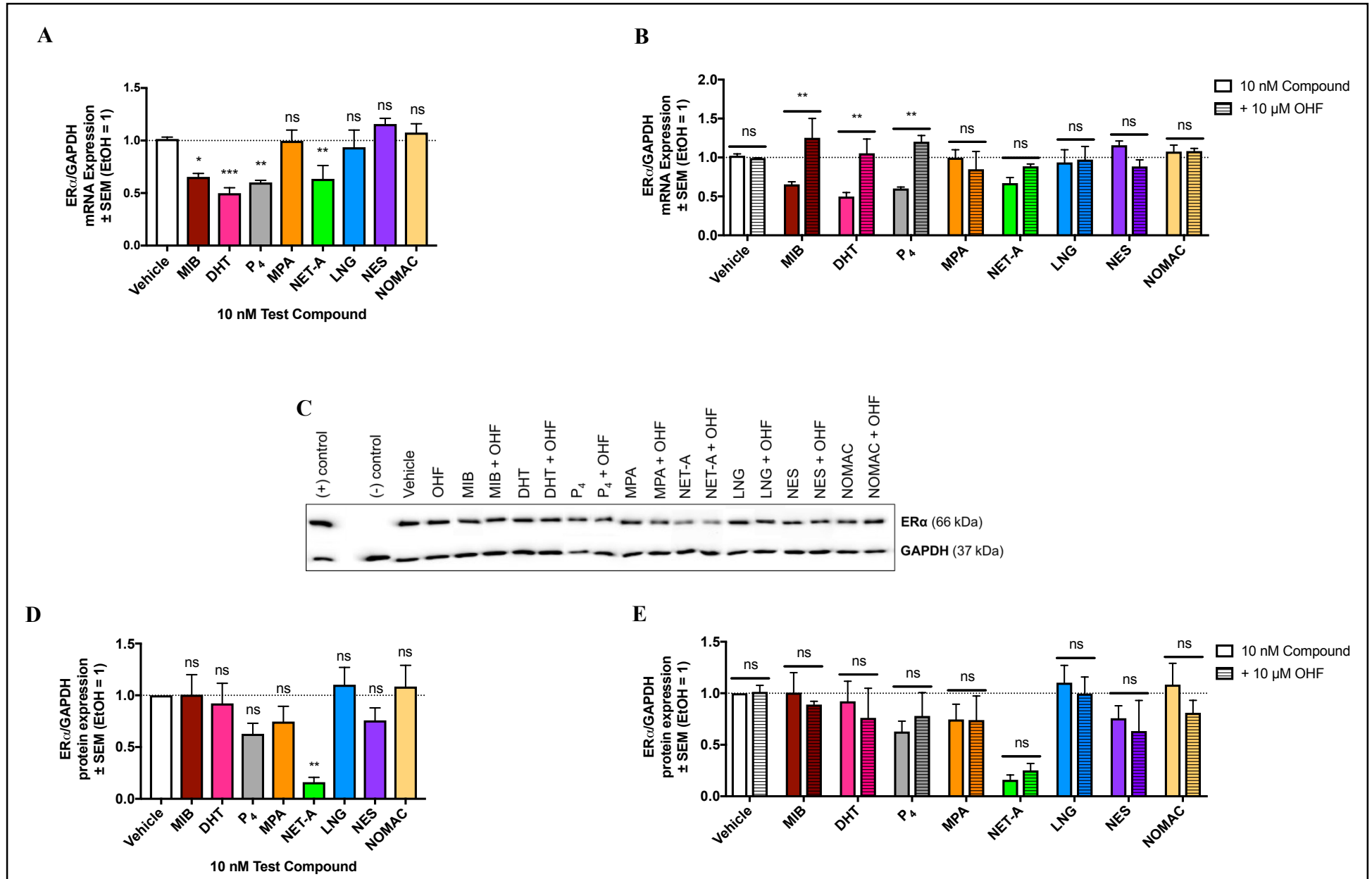
Figure 3.8 legend on next page.

Figure 3.8. Only NET-A downregulates both ERα mRNA and protein expression. MCF-7 BUS cells were treated for 24 hours with either 0.2% (v/v) EtOH (vehicle) or 10 nM Mib, DHT, P_4, MPA, NET-A, LNG, NES, or NOMAC, in the (A, D) absence and (B, E) presence of 10 µM OHF. (A, B) Total RNA was isolated, cDNA synthesized, and ERα and GAPDH mRNA expression analyzed using real-time qPCR. (C, D, E) Protein lysates were analyzed by western blotting using primary antibodies specific for ERα and GAPDH (loading control). HEK-293 cells transiently transfected with an expression vector for ERα was used as a positive control, while untransfected COS-1 cells were used as a negative control. (C) A representative western blot is shown. (D, E) ERα protein expression was quantified using the MyImageAnalysis™ software, and normalized to GAPDH expression. Results shown for qPCR and western blotting are the averages (± SEM) of at least three independent experiments. The ratio of ERα to GAPDH mRNA and protein expression is shown relative to the vehicle control. Statistical analysis was performed using (A, D) one-way ANOVA with Dunnett's (compares all columns vs. control column) as post-test or (B, E) two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

3.7 The MDA-MB-453 TNBC cell line expresses endogenous ERβ and AR.

Given that we were unable to detect ERβ mRNA in the MCF-7 BUS and T47D cells, we next used the MDA-MB-453 breast cancer cell line, a model system commonly used to investigate AR mechanisms in triple negative breast cancer (TNBC) (Hall *et al.*, 1994). Western blot analysis showed that the MDA-MB-453 cell line expresses the AR, GR and ERβ, but not PR-A, PR-B or ERα (Figure 3.9), suggesting that it is suitable model for investigating AR-mediated effects on ERβ (Vranic *et al.*, 2011).

However, as it is known that these cells express an AR with a point mutation in its LBD that has been shown to have reduced sensitivity to DHT and MPA in transactivation assays (Moore *et al*. 2012a), we investigated whether the progestogens would protect the mutant AR from degradation as was observed for the wild-type AR in the MCF-7 BUS cells (Figure 3.2). Western blot analysis showed that the androgens increase the protein levels of the mutant AR in the MDA-MB-453 cells, while the progestins had no effect (Figure 3.10B). Furthermore, results in Figure 3.10C show that the AR antagonist, OHF, inhibits the effects of Mib and DHT, while having no influence on the effects of the progestogens.
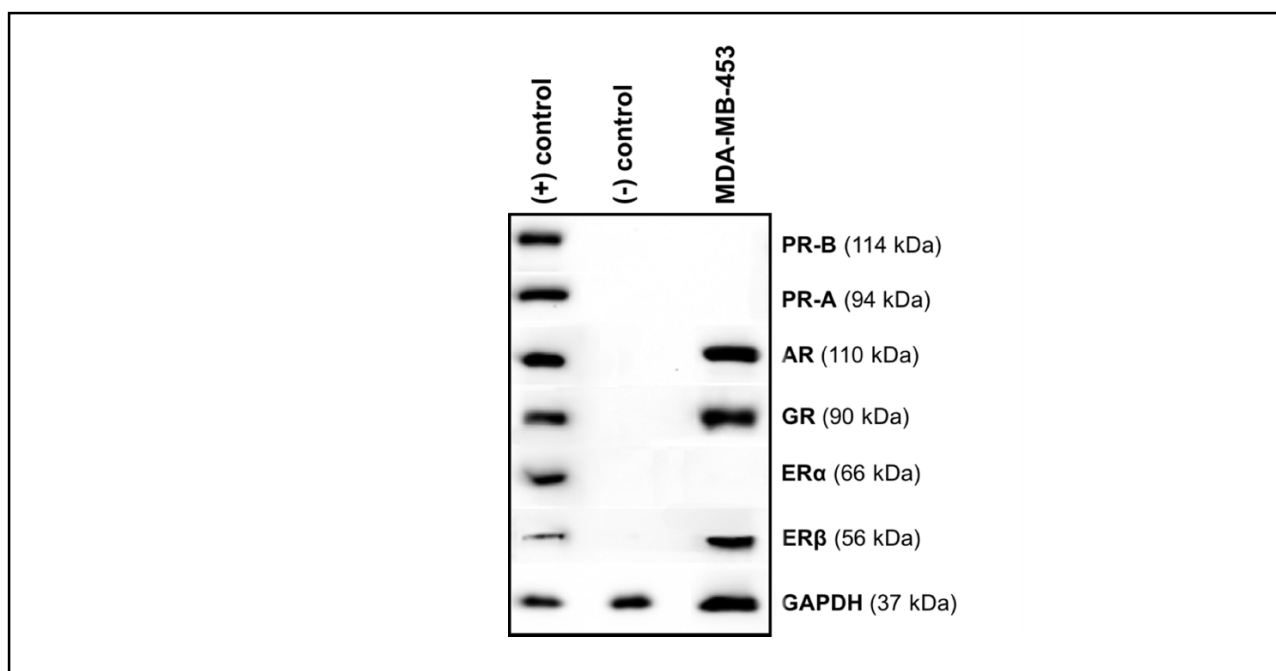
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Figure 3.9. The MDA-MB-453 TNBC cell line expresses endogenous ERβ and AR. Whole cell extracts were prepared from MDA-MB-453 breast cancer cells and analyzed by western blotting using primary antibodies specific for PR-A/B, AR, GR, ERα, ERβ and GAPDH (loading control). COS-1 cells transiently transfected with expression vectors for either PR-A, PR-B or GR, or HEK-293 cells transiently transfected with expression vectors for either the AR, ERα or ERβ, were used as positive controls. Untransfected COS-1 or HEK-293 cells were used as negative controls. A representative western blot of at least three independent experiments is shown.

3.8   Androgens increase ERβ mRNA expression in MDA-MB-453 breast cancer cells.

Considering that our results show that the MDA-MB-453 cell line expresses endogenous ERβ and AR, we next investigated whether ERβ mRNA and protein expression could be regulated by the androgens in the MDA-MB-453 breast cancer cell line. The results in Figure 3.11 showing that both Mib and DHT increased ERβ mRNA expression (Figure 3.11A) via the AR (Figure 3.11B), are consistent with the study by Rizza *et al.* (2014). We were unable to establish whether these androgens also increased ERβ protein expression, as the commercial ERβ antibody from Abcam (EPR3777) previously used to detect ERβ (Figure 3.4 and Figure 3.9) was discontinued. Western blot analysis using an alternate commercial ERβ antibody from Abcam (EPR20743) indicated that this antibody was not specific for ERβ, as a band of the same size as the ERβ protein was observed in the negative control (Figure B4, Addendum B). It is noteworthy that this new ERβ antibody (EPR20743) has subsequently also been discontinued.
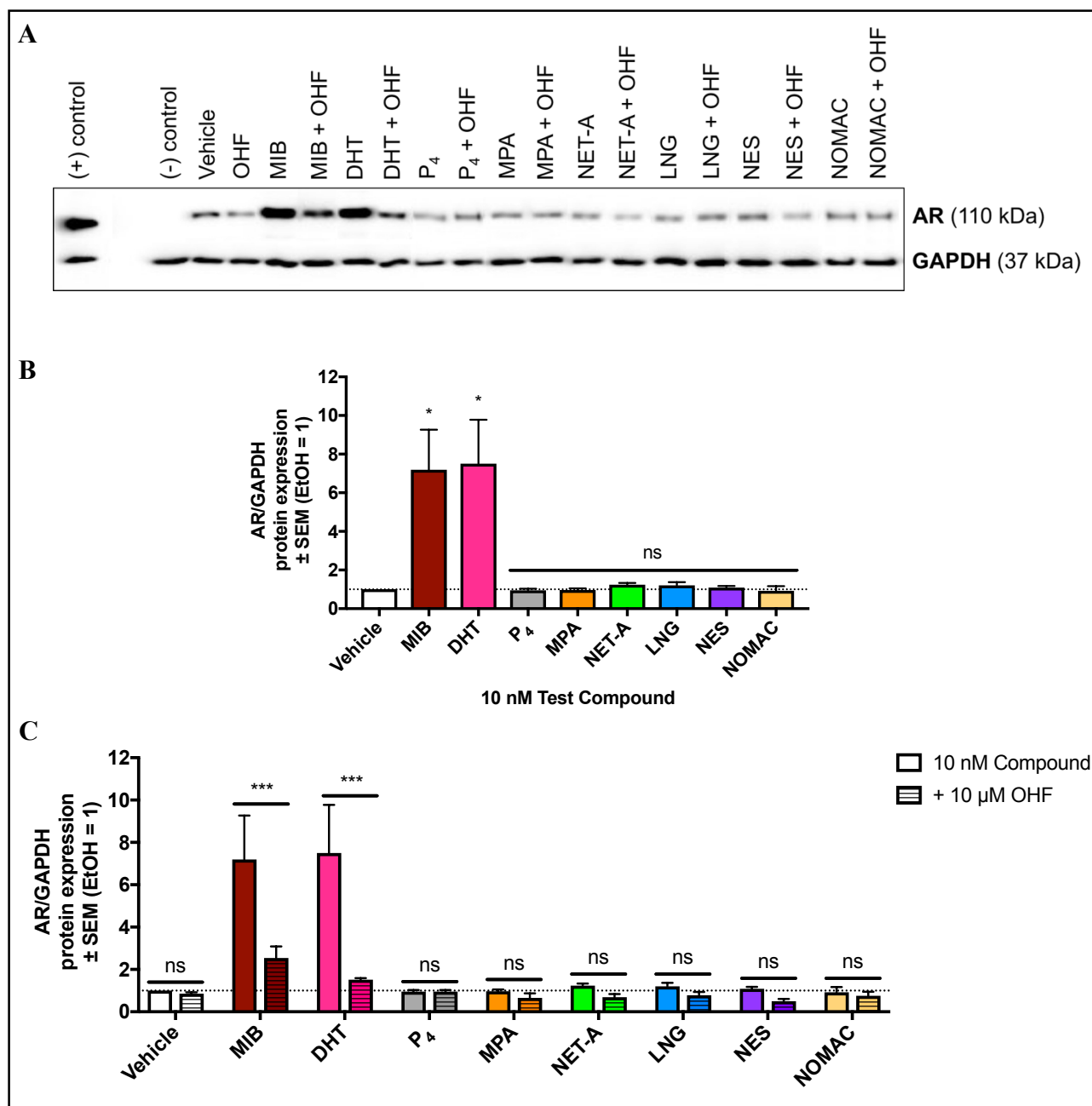
Figure 3.10. Both androgens induce stabilization of the endogenous mutant AR in the MDA-MB-453 breast cancer cell line. The human MDA-MB-453 breast cancer cells were treated for 24 hours with either 0.2% (v/v) EtOH (vehicle), or 10 nM Mib, DHT, P_4, MPA, NET-A, LNG, NES or NOMAC, in the absence and presence of 10 µM OHF. Protein lysates were analyzed by western blotting using primary antibodies specific for the AR and GAPDH (loading control). HEK-293 cells transiently transfected with an expression vector for the AR was used as a positive control, while untransfected COS-1 cells were used as a negative control. (A) A representative western blot of at least three independent experiments is shown. (B, C) AR protein expression was quantified using the MyImageAnalysis™ software, and normalized to GAPDH expression. Results shown are the averages (± SEM) of at least three independent experiments. The ratio of AR to GAPDH protein expression is shown relative to the vehicle control. Statistical analysis was performed using (B) one-way ANOVA with Dunnett's (compares all columns vs. control column) as post-test or (C) two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

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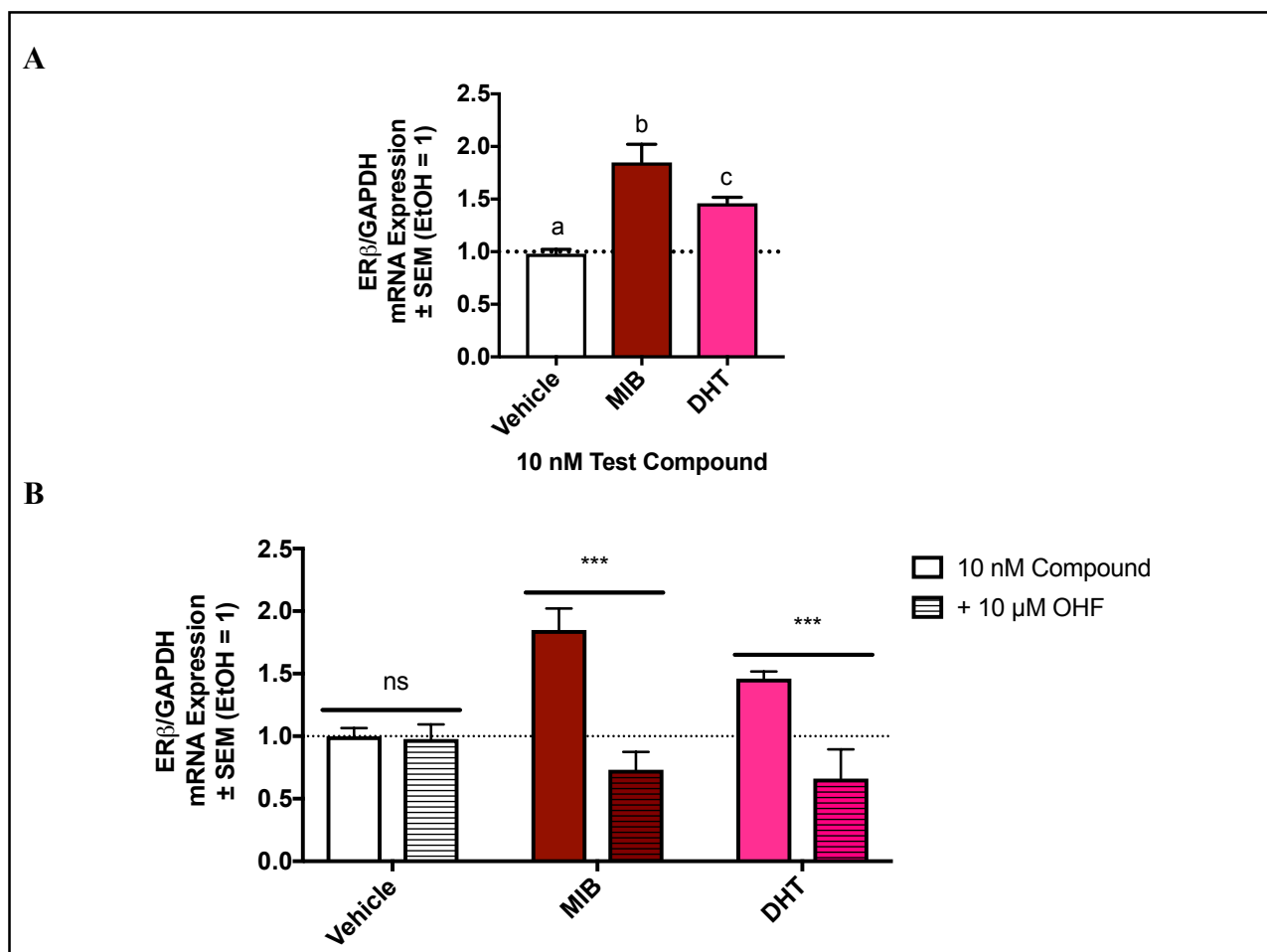**Figure 3.11. Mib and DHT differentially increased ERβ mRNA expression via the AR in MDA-MB-453 breast cancer cells.** The human MDA-MB-453 cells were treated for 24 hours with either 0.2% (v/v) EtOH (vehicle), 10 nM Mib or DHT, in the absence and presence of 10 μM OHF. Total RNA was isolated, cDNA synthesized, and ERβ and GAPDH mRNA expression analyzed using real-time qPCR. Results shown are the averages (± SEM) of at least three independent experiments. The ratio of ERβ to GAPDH mRNA expression is shown relative to the vehicle control. Statistical analysis was performed using (A) one-way ANOVA with Newman-Keuls (compares all columns to each other) as post-test or (B) two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

3.9 Like Mib and DHT, the androgenic progestins increase ERβ mRNA expression in MDA-MB-453 cells via an AR-mediated mechanism.

Next, we investigated whether progestins with androgenic properties, would increase ERβ mRNA expression in the MDA-MB-453 cells. Real-time qPCR results show that MPA and NET-A increased ERβ mRNA expression to the same extent as each other and DHT, while LNG increased expression to a greater extent than both Mib and DHT (Figure 3.12A). Results in Figure 3.12B show that the addition of the AR antagonist OHF reversed the MPA-, NET-A-, and LNG-induced effects on ERβ mRNA expression, suggesting that these progestins regulate ERβ mRNA expression via a mechanism

involving the AR. In contrast, NES and NOMAC, which display anti-androgenic properties similar to P$_4$, had no effect on ERβ mRNA expression (Figure 3.12A), while P$_4$ downregulated the expression (Figure 3.12A). However, unlike the results observed for P$_4$ on ERα mRNA expression, the AR was not required for the effects of P$_4$ on ERβ mRNA expression (Figure 3.12B). Due to the lack of a commercial antibody specific to ERβ, we were unable to evaluate the effects of the progestogens on ERβ protein expression.
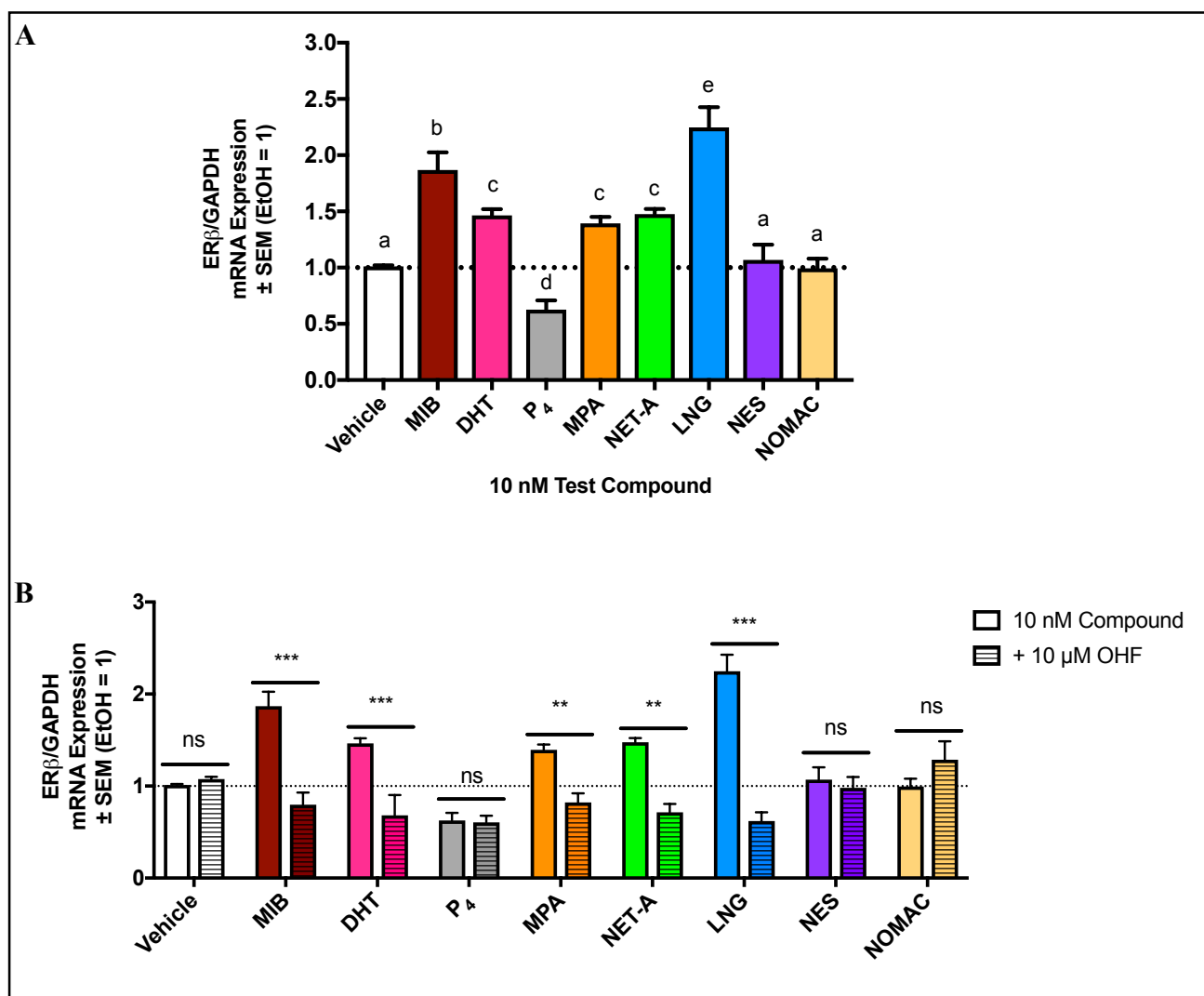
Figure 3.12. MPA, NET-A and LNG, like the androgens, upregulate ERβ mRNA expression via an AR-mediated mechanism in MDA-MB-453 cells. The human MDA-MB-453 cell line was treated for 24 hours with either 0.2% (v/v) EtOH (vehicle) or 10 nM Mib, DHT, P$_4$, MPA, NET-A, LNG, NES, or NOMAC, in the absence and presence of 10 μM OHF. Total RNA was isolated, cDNA synthesized, and ERβ and GAPDH mRNA expression analyzed using real-time qPCR. Results shown are the averages (± SEM) of at least three independent experiments. The ratio of ERβ to GAPDH mRNA expression is shown relative to the vehicle control. Statistical analysis was performed using (A) one-way ANOVA with Newman-Keuls (compares all columns to each other) as post-test or (B) two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

3.10   DHT decreases E_2-induced proliferation of the MDA-MB-453 breast cancer cell line when ERα and ERβ are co-expressed.

Given that the MDA-MB-453 cell line expresses ERβ and the AR, we next evaluated proliferation of these cells in response to E_2, in the absence and presence of DHT. Results from the MTT cell viability assay show that E_2 dose-dependently increased MDA-MB-453 breast cancer cell proliferation, and that DHT had no effect on the E_2-induced proliferation (Figure 3.13A). As it has previously been shown that DHT inhibits E_2-induced proliferation in MCF-7 breast cancer cells expressing both ERα and ERβ (Ando *et al.*, 2002), we next transiently transfected the MDA-MB-453 cells with an expression vector encoding the human ERα. The results in Figure 3.13C show that DHT decreased the E_2-induced proliferation of the MDA-MB-453 cells co-expressing ERα and ERβ. This decrease appeared to be more pronounced in cells transiently transfected with both ERα and ERβ (Figure 3.13E). Results from similar experiments conducted with the progestins were inconclusive as the E_2 control did not cause proliferation in this experiment (data not shown). Importantly, these results are from a single experiment performed in triplicate and should be repeated to determine the statistical significance of the findings.

A — Endogenous ERβ

B

C — Endogenous ERβ + over-expressed ERα

D

E — Over-expressed ERα + endogenous or over-expressed ERβ

F
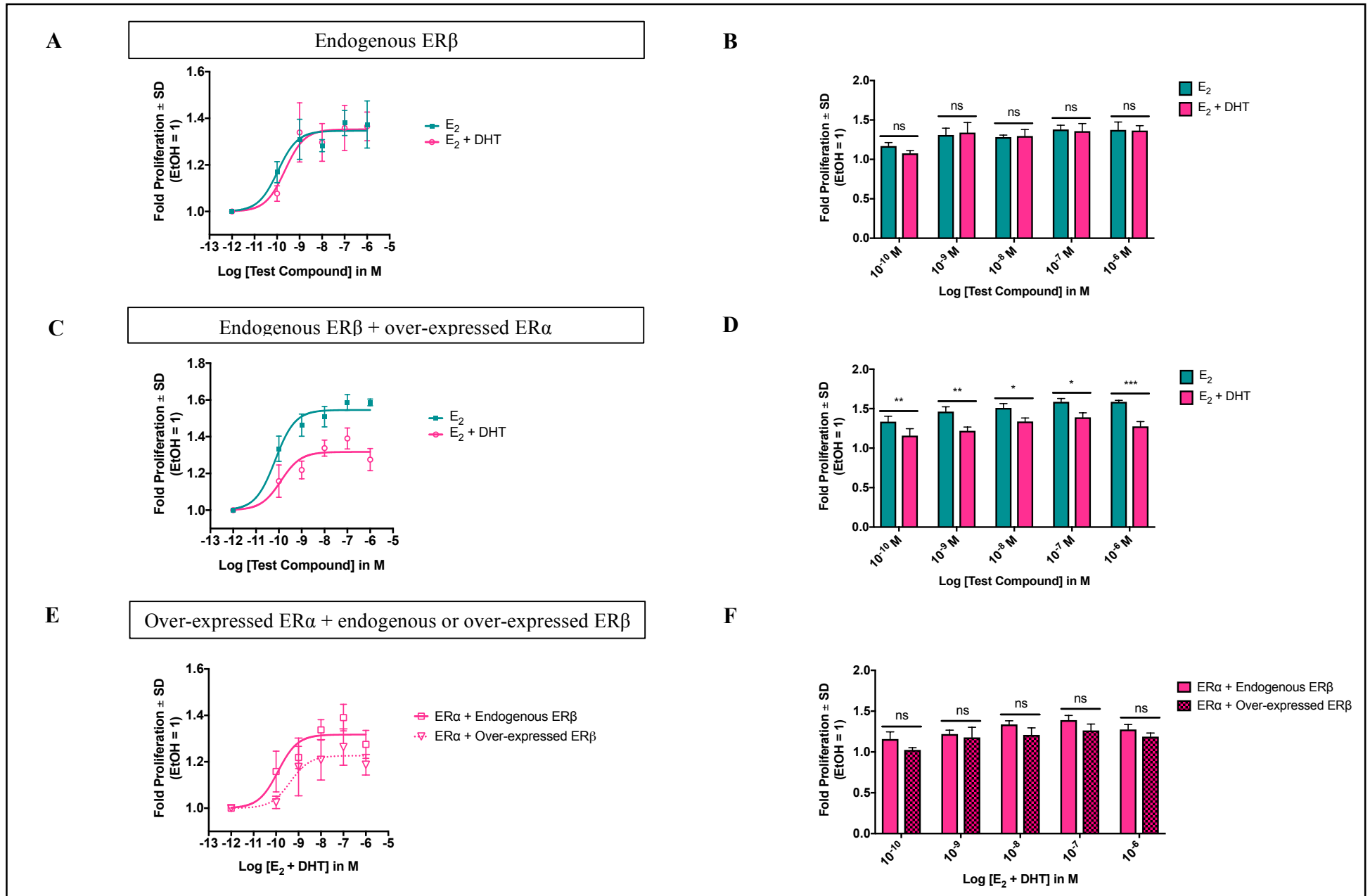
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Figure 3.13. E$_2$-induced proliferation of MDA-MB-453 breast cancer cells is decreased in the presence of DHT when ERα and ERβ are co-expressed. The human MDA-MB-453 breast cancer cells were either (A) untransfected, or transiently transfected with an expression vector for human ERα in the (C) absence and (E) presence of an expression vector for human ERβ. All cells were treated for 72 hours with either 0.2% (v/v) EtOH (vehicle) or increasing concentrations of E$_2$, in the absence and presence of equimolar concentrations of DHT. The medium was aspirated and cells were re-treated for an additional 44 hours. Cell proliferation was measured using the MTT cell viability assay. Results are represented as fold proliferation relative to the vehicle control. The results shown are the averages (± SD) of a single experiment with each condition performed in triplicate. (B, D, F) The data from (A), (C) and (E) were re-plotted to show the statistical significance between either E$_2$ vs. E$_2$ plus DHT, or over-expressed ERα and endogenous ERβ vs. over-expressed ERα and over-expressed ERβ. Statistical analysis was performed using two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

Chapter 4

Discussion

4.1   Introduction

Multiple steroid receptors play a role in breast cancer pathogenesis and often influence each other's activity (reviewed in (Sikora 2016)). For example, ERβ decreases breast cancer cell growth by inhibiting the activity of ERα when the ER subtypes are co-expressed, while ERβ mimics the proliferative effects of ERα in ERα-negative breast cancer (Leygue *et al.*, 1998; Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999; Williams *et al.*, 2008; Zhao *et al.*, 2008; Hartman *et al.*, 2009; Leygue and Murphy, 2013). Similarly, the AR, which is most frequently co-expressed with ERα in breast cancer (Agoff *et al.*, 2003; Niemeier *et al.*, 2010; Tsang *et al.*, 2014), has also been shown to decrease breast cancer cell growth in ER-positive breast cancer (Peters *et al.*, 2009; Rizza *et al.*, 2014). At least two mechanisms have been reported for this inhibitory role of the AR in ER-positive breast cancer (Peters *et al.*, 2009; Rizza *et al.*, 2014). One mechanism is that the androgen-bound AR competes with ERα for binding to EREs in ER target genes (Peters *et al.*, 2009). A second mechanism is that the androgen-activated AR binds to an ARE in the ERβ gene promoter, resulting in increased expression of the anti-proliferative ERβ (Rizza *et al.*, 2014). Interestingly, inhibition of ERα transcriptional activity has also been shown when the AR was activated by the first generation progestin, MPA (Peters *et al.*, 2009). However, MPA has also been shown to promote the development of breast cancer by a mechanism involving the disruption of normal androgen signaling (Carroll *et al.*, 2016). In fact, other progestins commonly used in contraception and menopausal HT, such as NET-A and LNG, have also been shown to increase breast cancer risk (Rossouw *et al.*, 2002; Beral *et al.*, 2003; Fournier *et al.*, 2008). Although progestins were designed to mimic the activity of P_4 by binding to the PR, many progestins elicit off-target biological effects by binding to other steroid receptors (Hapgood *et al.* 2004; Sitruk-Ware 2006; Africander *et al.* 2011a; Stanczyk *et al.* 2013). Particularly relevant to this thesis is the fact that some progestins bind to the AR, and either elicit androgenic properties similar to the natural androgen, DHT, or anti-androgenic properties similar to the natural progestogen, P_4 (Africander *et al.* 2014; Louw-du Toit *et al.* 2017a). The question that arises is whether progestins displaying androgenic properties activate the AR by a similar mechanism as DHT, and whether these progestins, like DHT, would increase ERβ expression. Whether the androgen- or progestin-activated AR also modulates ERα expression is not known. In Chapter 3, we thus addressed these questions by investigating the ability of the progestins to either induce the AR N/C interaction or inhibit the DHT-induced N/C interaction. Effects of the progestins, relative to each other, P_4, DHT and the synthetic androgen Mib, on ERα and ERβ expression, as well as breast cancer cell proliferation, was also investigated.

4.2   Progestins binding to the AR elicit different conformations in the receptor.

It is well-known that the conformation induced in a steroid receptor by a particular ligand contributes to the biological activity of that ligand (Griekspoor *et al.*, 2007). For the AR, it was previously thought that all AR agonists induce the N/C interaction, while AR antagonists inhibit the androgen-induced AR N/C interaction (Langley *et al.*, 1995). However, our group and others have shown that although MPA and NET-A elicit similar agonist activity to DHT (Africander *et al.* 2014; Louw-du Toit *et al.* 2017a), MPA does not induce the AR N/C interaction, while DHT and NET-A do (Kemppainen *et al.*, 1999; Tanner, 2002; Africander *et al.*, 2014). It was thus suggested that MPA and NET-A activate the AR by inducing different conformations in the receptor (Africander *et al.*, 2014). The first aim of this thesis was thus to investigate the ability of selected progestins to induce the AR N/C interaction, or antagonize the DHT-induced AR N/C interaction, as a measure of receptor conformation (Section 3.1). The results in Figure 3.1A showing that P_4 and MPA do not induce the AR N/C interaction, while NET-A does, are consistent with a previous study from our group (Africander *et al.*, 2014). The lack of AR N/C interaction with MPA is possibly due to the fact that this first generation progestin is a 17α-hydroxyprogesterone derivative (Schindler *et al.*, 2003) that is more structurally related to P_4 (Schindler *et al.*, 2003). Similarly, NES and NOMAC, which are 19-norpregnane progestins that are also structurally related to P_4 (Schindler *et al.*, 2003), did not induce the AR N/C interaction (Figure 3.1A). In addition, the spironolactone derivative, DRSP, previously shown to have a similar biochemical profile as P_4 (Krattenmacher, 2000), also did not induce the AR N/C interaction. We show for the first time that LNG and GES induce the AR N/C interaction to the same extent as each other and NET-A, suggesting that these progestins activate the AR by a similar mechanism as NET-A (Figure 3.1A). This may be due to the fact that these progestins are all 19-nortestosterone derivatives, which are structurally related to the natural androgen, testosterone (Schindler *et al.*, 2003). The fact that NET-A, LNG and GES have previously been shown to display similar potencies and efficacies as DHT, but do not induce the AR N/C interaction to the same extent as DHT, underlines the fact that the N/C interaction does not predict ligand potency and efficacy for transactivation (Africander *et al.*, 2014). Taken together, these results are in agreement with previous reports that structural differences between ligands can lead to differences in receptor conformation (Kuil *et al.*, 1995).

When investigating whether progestins could antagonize the DHT-induced AR N/C interaction, the results showed that, P_4, NES, NOMAC and DRSP do not antagonize the AR N/C interaction induced by DHT (Figure 3.1B). Interestingly, P_4 has previously been shown to inhibit the DHT-induced AR N/C interaction (Kemppainen *et al.*, 1999). It is possible that the difference between our results and

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that of Kemppainen and co-workers can be ascribed to the use of different experimental conditions. For example, hormones are known to elicit cell-specific effects (reviewed in (Katzenellenbogen *et al.* 1996)), and Kemppainen *et al.* (1999) used the Chinese hamster ovary (CHO) cell line as an *in vitro* model system, while we used COS-1 African monkey kidney fibroblast cells. In addition, we used the pTAT-GRE-E1b-luc luciferase reporter construct that has two copies of the GRE (Sui *et al.*, 1999), while Kemppainen used the G5E1b-luc construct that has five copies of the GRE (Lillie and Green, 1989). Consistent with a previous study (Tanner, 2002), our results showed that MPA, but not NET-A, repressed the DHT-induced AR N/C interaction (Figure 3.1B). This result suggests that inhibition of the androgen-induced AR N/C interaction does not imply AR antagonist activity. As expected, like NET-A, LNG and GES, did not repress the DHT-induced AR N/C interaction, suggesting that LNG and GES elicit similar conformations in the AR as NET-A. Taken together, our results showed that the AR N/C interaction is not induced by all AR agonists, nor is the DHT-induced N/C interaction inhibited by all AR antagonists. Importantly, the mammalian two-hybrid assay does not include the full-length human AR, nor does it take into account the conformational changes induced in the receptor by its interaction with DNA and other proteins of the transcription machinery (Griekspoor *et al.*, 2007; Africander *et al.*, 2014). Thus, the results from this *in vitro* mammalian two-hybrid assay may not reflect the progestogen-induced AR conformation *in vivo*.

4.3   Progestogens do not influence AR protein levels in MCF-7 BUS breast cancer cells.

The evidence in the literature suggests that the stability of the AR protein is increased by the binding of androgens, while anti-androgens cause AR protein degradation (Kemppainen *et al.*, 1992; Zhou *et al.*, 1995; Greeve *et al.*, 2004). We therefore investigated whether progestins displaying androgenic activity would stabilize the AR protein in the MCF-7 BUS cells in a similar manner as previously reported for the synthetic androgen Mib and the natural androgen DHT (Ando *et al.*, 2002; Greeve *et al.*, 2004; Macedo *et al.*, 2006). Both Mib and DHT increased AR protein levels (Figure 3.2B), suggesting that the androgens stabilize the AR. In contrast, P_4 and the progestins that can bind to the AR do not cause degradation of the AR, suggesting that these ligands induce a conformation in the AR LBD that protects it from being degraded (Kemppainen *et al.*, 1999; de Jésus-Tran *et al.*, 2006). Importantly, it has previously been shown that the AR is required for the effects of MPA in breast cancer therapy (Birrell *et al.* 1995a), and a reduction in AR protein levels has been shown to contribute to the failure of MPA therapy in breast cancer tumours (Buchanan *et al.*, 2005). The fact that neither MPA, nor NET-A or LNG, decreased AR protein levels in our experimental systems, suggest that these progestins may in fact be effective in breast cancer therapy. However, these results

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need to be interpreted with caution as MPA, NET-A and LNG have all been associated with increased risk of breast cancer.

4.4 The MPA-, NET-A- and LNG-induced increase in T47D human breast cancer cell proliferation requires multiple steroid receptors.

Numerous experimental studies have investigated the effects of the androgens and progestins on breast cancer cell proliferation, but the results are often contradictory. The latter may be due to factors such as intra-laboratory cell line heterogeneity or phenotypic drift (Burdall *et al.*, 2003), or the fact that few studies directly compare the effects of these hormones in the same model system. We thus directly compared the effects of selected progestins relative to each other, P_4 and the androgens, Mib and DHT, on proliferation of the commonly used MCF-7 BUS and T47D breast cancer cell lines. Our results showing that Mib and DHT increased proliferation of both these cell lines (Figure 3.3) are consistent with some studies (Hackenberg *et al.* 1988; Birrell *et al.* 1995b; Baniwal *et al.* 2011), but not others showing inhibition of proliferation of the MCF-7 and T47D breast cancer cell lines (Birrell *et al.* 1995b; Ando *et al.* 2002; Aspinall *et al.* 2004; Peters *et al.* 2009; Lanzino *et al.* 2013; Rizza *et al.* 2014). Similarly, our result showing that P_4 and the selected progestins increased proliferation of both MCF-7 BUS and T47D breast cancer cell lines (Figure 3.3) are in agreement with numerous other studies (Jeng and Jordan, 1991; Van Der Burg *et al.*, 1992; Jeng *et al.*, 1992; Catherino *et al.*, 1993; Cappelletti *et al.*, 1995; Schoonen *et al.*, 1995a, 1995b; Franke and Vermes, 2003; Liang *et al.*, 2006; Giulianelli *et al.*, 2012; Ruan *et al.*, 2012), but in contrast to other studies (Jeng and Jordan, 1991; Jeng *et al.*, 1992; Botella *et al.*, 1994; Formby and Wiley, 1998; Wiebe *et al.*, 2000; Ruan *et al.*, 2012). Discrepancies in responses between the various studies may be due to the use of different cell lines, such as the T47D vs. MCF-7 vs. the MCF-7 BUS cells or different concentrations of progestins used (reviewed in (Moore *et al.* 2012b)).

Progestins elicit their biological effects by binding to the PR but also other members of the steroid receptor family (reviewed in (Africander *et al.* 2011a)). As some progestins bind to multiple steroid receptors, we investigated the role of the AR, PR, GR and ER, which are all endogenously expressed in both the MCF-7 BUS and T47D cells (Figure 3.4) (Horwitz *et al.*, 1975, 1978; Vladusic *et al.*, 2000; Singer *et al.*, 2003). Considering that Bical, a well-known antagonist of androgen effects via the AR, did not reverse the effects of the androgens on cell proliferation in T47D cells (Figure B6, Addendum B), we used the AR antagonist, OHF, instead. In the MCF-7 BUS cells, our results showed that the AR was required for the effects of MPA, NET-A and LNG (Figure 3.5A). However, using RU486, a well-known PR, GR but also AR antagonist (Spitz and Bardin, 1993; Africander *et al.*,

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2014), we could not draw any conclusions about the role of the PR or GR in these cells. This was due to the fact that RU486 did not antagonize the effects of the GR-specific agonist, Dex, or PR-specific agonist, R5020, while also increasing proliferation. These results suggest that RU486 acts as an agonist, rather than an antagonist in the MCF-7 BUS cells (Figure B2, Addendum B). It has previously been reported that RU486 acts as an agonist in some cells, while it is an antagonist in others (Catherino *et al.*, 1993; Spitz and Bardin, 1993; Kalkhoven *et al.*, 1994). The role of the ER in the MCF-7 BUS cells could also not be investigated as 1 µM ICI significantly decreased the viability of the MCF-7 BUS cells (Figure B3, Addendum B), suggesting that this concentration of ICI may be cytotoxic to these cells.

In the T47D cells, both the AR (Figure 3.5B) and PR (Figure 3.5D) were shown to be required for the effects of MPA, NET-A and LNG, while the ER was required for the effects of NET-A and LNG (Figure 3.5F). RU486 did not inhibit effects of the GR-specific agonist, Dex, in the T47D cells, suggesting that it cannot act as an antagonist of the GR in this cell line (Figure B2, Addendum B). RU486 is known to act as either an agonist or an antagonist, depending on receptor density (Zhao *et al.*, 2003). No conclusions could thus be drawn about the role of the GR in the responses of MPA, NET-A and LNG. However, as it is well-known that MPA is an agonist of the GR, while NET-A and LNG are not (Koubovec *et al.* 2005; Africander *et al.* 2011b; Stanczyk *et al.* 2013), one could speculate that MPA may increase proliferation of the T47D or MCF-7 BUS cells via the GR. Nonetheless, these results suggest that multiple steroid receptors are required for the effects of MPA, NET-A and LNG on T47D breast cancer cell proliferation. It is noteworthy that at least one other study has previously shown that the proliferative effects of LNG occurs via the ER in MCF-7 breast cancer cells (Catherino *et al.*, 1993). Although ICI is an antagonist of both ERα and ERβ, it is likely that the proliferative effects of NET-A and LNG are via ERα as both these progestins have been shown to bind to ERα, but not ERβ (Louw-du Toit *et al.* 2017a). Considering that the T47D cells express higher PR levels than the MCF-7 cells (Janowski *et al.*, 2006), it is not surprising that this steroid receptor was required for progestogen-induced proliferation. Considering that progestins were designed to act via the PR (Sitruk-Ware, 2006), it is likely that the PR may in fact also be required for the progestogen-induced proliferation in the MCF-7 BUS cells. Taken together, we showed that the AR, PR and ER are required for the effects of MPA, NET-A and LNG in the T47D cell line, while only the AR was required for their effects in the MCF-7 BUS cell line.

4.5   Mib and DHT differentially increase ERβ mRNA expression in MCF-7 BUS and T47D breast cancer cells.

A recent study by Rizza *et al.* (2014) was the first to show that Mib and DHT both increased the expression of ERβ mRNA and protein in MCF-7 breast cancer cells via a mechanism involving the AR. We validated these findings on mRNA expression in the human MCF-7 BUS breast cancer cells, which is a clone of the MCF-7 breast cancer cell line that is more estrogen-sensitive (Villalobos *et al.*, 1995), as well as the human T47D cell line. Subsequent to two biological replicate experiments showing that both Mib and DHT increased ERβ mRNA expression in the MCF-7 BUS (Figure 3.6A) and T47D (Figure 3.6B) breast cancer cell lines, we were unable to reproduce the above-mentioned results. Therefore, neither the role of the AR in mediating these responses on ERβ mRNA expression, nor effects on ERβ protein expression, could be detected. The exact reason for the inability to detect ERβ mRNA in these cell lines in subsequent experiments is not clear. However, at least one study has reported that ER levels in MCF-7 cells can vary between experiments (Osborne *et al.*, 1987). Furthermore, it has previously been reported that human breast cancer cell lines are prone to phenotypic drift, which may result in loss of steroid receptor expression when cells undergo more than ten passages (Wistuba *et al.*, 1998; Masters, 2000). Thus, as the MCF-7 BUS and T47D breast cancer cell lines used in this thesis were passaged up to thirty-five times, it is likely that the expression of ERβ may have been lost (reviewed in (Masters 2000)).

4.6   Androgens decrease ERα mRNA via the AR in the MCF-7 BUS breast cancer cell line.

The study by Rizza *et al.* (2014) investigated androgen effects on only ERβ expression. Considering that ERα and ERβ play opposing roles in breast cancer (Kuiper *et al.*, 1996; Leygue *et al.*, 1998; Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999; Zhao *et al.*, 2008; Leygue and Murphy, 2013), it is important to also investigate the effects of Mib and DHT on ERα expression. One study, more than 20 years ago, showed that prolonged exposure (i.e. up to 14 days) to 10 nM DHT caused downregulation of ERα mRNA and protein expression via the AR in the ZR-75-1 human breast cancer cell line (Poulin *et al.*, 1989). Interestingly, this cell line is known to express higher levels of AR than the MCF-7 breast cancer cell line (Birrell *et al*. 1995b). As it is well-known that hormones can elicit cell-specific effects (reviewed in (Katzenellenbogen *et al*. 1996)), we next investigated the effects of Mib and DHT on ERα expression in the same *in vitro* model system as for ERβ expression, namely the MCF-7 BUS breast cancer cell line. Consistent with the study by Poulin *et al*. (1989), our results showed that the natural androgen, DHT, as well as the synthetic androgen, Mib, significantly decreased ERα

mRNA expression (Figure 3.7A) via the AR (Figure 3.7B). However, the result showing that Mib and DHT had no effect on ERα protein expression in the MCF-7 BUS cells (Figure 3.7D) is in contrast to the effects shown in the ZR-75-1 cell line (Poulin *et al.*, 1989). The discordance of our results between ERα mRNA and protein expression suggests that post-translational modifications may have occurred in the MCF-7 BUS cell line, as previously suggested by Liu and co-workers (Liu *et al.*, 2016). Although the precise mechanism whereby the AR downregulates ERα mRNA expression is not known, it may be that the Mib- and DHT-activated AR decrease ERα mRNA expression via the ERα tethering to DNA-bound transcription factors like AP-1 (McDonnell and Norris, 2002). This is likely as both Mib and DHT have been shown to repress an AP-1-luc reporter construct via the AR (Africander *et al*. 2014; Louw-du Toit *et al*. 2017a), and it is known that AP-1 *cis*-elements are found in the promoter of the ERα gene (Carroll *et al.*, 2006).

When investigating the effects of the androgenic progestins on ERα expression, we showed that NET-A decreased ERα mRNA and protein expression, while MPA and LNG had no effect (Figure 3.8A and D). In contrast to the effects of Mib and DHT on the mRNA expression of ERα, our results showed that the effect of NET-A on ERα mRNA (Figure 3.8B) and protein (Figure 3.8E) expression was not via the AR. This result was unexpected as we had previously shown that the AR is required for the effects of NET-A on cell proliferation (Figure 3.5A and Figure 3.5B). Although NET-A has been shown to bind to ERα, it displays a much lower affinity for ERα than the AR (Louw-du Toit *et al*. 2017a). Thus, it is unlikely that NET-A will elicit its physiological effects via ERα in a system where both ERα and the AR are expressed. Much to our surprise, our results showed that P_4 downregulated ERα mRNA expression (Figure 3.8A) via the AR (Figure 3.8E). This result is hard to interpret as P_4 is an antagonist for AR transactivation via an ARE, but is a partial agonist for AR transrepression via either AP-1 or NFκB-luc reporter plasmids (Africander *et al*. 2014; Louw-du Toit *et al*. 2017a). Thus, it is likely that the P_4-bound AR could decrease ERα mRNA expression via a similar tethering mechanism as suggested above for Mib and DHT.

4.7 Androgens as well as progestins with androgenic properties increase ERβ mRNA expression via an AR-mediated mechanism in the human MDA-MB-453 breast cancer cell line.

As we were unable to detect ERβ mRNA in the MCF-7 BUS and T47D cells, an alternative *in vitro* model system was required for the investigation into the effects of the androgenic hormones on ERβ expression. Thus, the MDA-MB-453 human breast cancer cells, a cell line commonly used to investigate AR mechanisms in TNBC (Hall *et al.*, 1994), was characterized in terms of steroid

receptor expression. Our results confirmed previous reports that this cell line expresses endogenous AR, GR and ERβ, but not PR-A, PR-B or ERα (Figure 3.9) (Hall *et al*. 1994; Al-Bader *et al*. 2011; Vranic *et al*. 2011; Moore *et al*. 2012a; Barton *et al*. 2017). It is noteworthy that this cell line expresses a mutant AR with a point-mutation in the LBD (Moore *et al*. 2012a). This mutant AR has been shown to have reduced sensitivity to DHT and MPA in transactivation assays (Moore *et al*. 2012a). While DHT has been shown to stabilize the mutant AR in the MDA-MB-453 breast cancer cells in a similar manner to the wild-type AR (Yeap *et al.*, 1999; Ni *et al.*, 2011), this has not been shown for MPA or any other progestins displaying androgenic properties. Knowing that P_4 and the selected progestins protect the wild-type AR from degradation in the MCF-7 BUS cells (Figure 3.2), effects on the mutant AR were also investigated. Consistent with our results for the wild-type AR in the MCF-7 BUS cells (Figure 3.2), we showed that neither P_4 nor any of the progestins influenced the protein levels of the mutant AR (Figure 3.10B). Mib and DHT, however, increased the protein levels of the mutant AR in the MDA-MB-453 cells (Figure 3.10B), while the AR antagonist, OHF, reversed these effects (Figure 3.10C). Taken together, our results showed that the androgens and progestins that bind to the AR had similar effects on the protein levels of the mutant and wild-type AR, suggesting that the point mutation in the LBD of the AR mutant expressed in the MDA-MB-453 cell line does not influence the activity of these hormones in this cell line.

We next hypothesized that ERβ expression would be regulated by the androgens in the MDA-MB-453 breast cancer cell line. Indeed, our results confirmed that both Mib and DHT increased ERβ mRNA expression (Figure 3.11A) via the AR (Figure 3.11B) in the MDA-MB-453 cells. MPA, NET-A and LNG also increased ERβ mRNA expression (Figure 3.12A) via the AR (Figure 3.12B), while NES and NOMAC, which display anti-androgenic properties similar to P_4, did not regulate ERβ mRNA expression (Figure 3.12A). LNG has previously been shown to bind to the AR with similar affinity as DHT, and display similar AR agonist potency for transactivation as DHT, MPA and NET-A (Louw-du Toit *et al*. 2017a). Thus, it was a surprise that LNG increased ERβ mRNA expression to a greater extent than both Mib and DHT. Due to the unavailability of a reliable ERβ antibody, effects on ERβ protein expression could not be investigated. In fact, numerous studies have reported that multiple commonly used commercial ERβ antibodies greatly vary in sensitivity and specificity for this protein (Choi *et al.*, 2001; Skliris *et al.*, 2001; Carder *et al.*, 2005; Weitsman *et al.*, 2006; Wu *et al.*, 2012; Andersson *et al.*, 2017; Nelson *et al.*, 2017). For example, Nelson and co-workers have shown that some ERβ antibodies detect either a band in ERβ-negative samples, implying non-specificity, or additional non-specific proteins of the incorrect size, implying cross-reactivity with other proteins and reduced sensitivity for ERβ (Nelson *et al.*, 2017). Moreover, it has been shown

that some commercial ERβ antibodies cross-react with ERα (Wu *et al.*, 2012). The lack of a reliable commercially available ERβ antibody has prevented the evaluation of effects on ERβ protein expression in this study, and highlights the need for a reliable ERβ antibody to fully understand the role of ERβ in breast cancer.

4.8 DHT decreases E$_2$-induced proliferation of the MDA-MB-453 breast cancer cell line when ERα and ERβ are co-expressed.

As mentioned in Section 4.6, ERα and ERβ, play differential roles in breast cancer, with ERα being the driver of proliferation, while the role of ERβ is dependent on whether ERα is expressed (Leygue *et al.*, 1998; Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999; Zhao *et al.*, 2008). In the absence of ERα, ERβ has been shown to increase proliferation and mimic the effects of ERα, while ERβ antagonizes the proliferative effects of ERα when these receptors are co-expressed (Moore *et al.*, 1988; O'Neill *et al.*, 2004; Skliris *et al.*, 2006; Fox *et al.*, 2008; Leygue and Murphy, 2013). As the MDA-MB-453 TNBC cells do not express ERα, the results in Figure 3.13A showing increased proliferation in the presence of E$_2$, suggest that the proliferative response is via the endogenous ERβ. Interestingly, E$_2$-induced proliferation was decreased when ERα and ERβ were co-expressed (Figure 3.13C and Figure B5, Addendum B), demonstrating that ERβ has a bi-faceted role in breast cancer cell proliferation. Our result showing that DHT decreased E$_2$-induced proliferation of the MDA-MB-453 cells when the ER subtypes are co-expressed (Figure 3.13C) is consistent with a previous study (Ando *et al.*, 2002). These authors showed that DHT inhibited the proliferation of MCF-7 human breast cancer cells in the absence and presence of E$_2$, and that the effect required the AR (Ando *et al.*, 2002). Thus, although we did not evaluate the role of the AR in the DHT-induced effect, it is likely that DHT elicits its growth inhibitory effects by activating the AR. In addition, the over-expression of ERβ appears to enhance the DHT-induced decrease in E$_2$-mediated proliferation (Figure 3.13E). It can thus be speculated that an increase in ERβ mRNA expression by the androgen-activated AR may lead to anti-proliferative effects in cells co-expressing ERα and ERβ. Similar effects may thus be observed with progestins displaying androgenic properties comparable to DHT, as our results have shown that all these compounds increased ERβ mRNA expression via the AR in MDA-MB-453 cells. However, as these results are from a single experiment with each condition performed in triplicate, these experiments need to be repeated before definitive conclusions can be made.

4.9 Conclusion

Although progestins were designed to mimic the biological activity of P$_4$ by binding to the PR, the findings of this thesis, summarized in Table 4.1 and 4.2, highlight the fact that these compounds do

not always mimic the actions of P_4 at a molecular level. In this thesis, the focus was on progestins that bind to the AR and elicit either androgenic activity like DHT, or anti-androgenic activity like P_4. Our results showed that although MPA, NET-A and LNG are as potent and efficacious as DHT, these progestins do not necessarily activate the AR by the same mechanism as DHT. Nonetheless, like DHT, these three androgenic progestins increased ERβ mRNA expression via the AR in the MDA-MB-453 breast cancer cell line. Although results could not be reproduced after two experiments, likely due to loss of ERβ expression due to high passage numbers, we showed that both Mib and DHT also increased ERβ mRNA expression in the ERα-positive MCF-7 BUS and T47D cells. It is thus possible that MPA, NET-A and LNG would also increase ERβ mRNA expression in ERα-positive breast cancer cells. While the Mib- and DHT-activated AR decreased ERα mRNA expression, MPA, NET-A and LNG did not influence ERα expression. The result showing downregulation of ERα mRNA expression by the androgen-activated AR suggests a third mechanism for the inhibitory role of the AR in ER-positive breast cancer. However, this mechanism is only true when the AR is activated by the classical androgens, and not when activated by progestins displaying androgenic properties. On the other hand, these results should be interpreted with caution as the AR-mediated effects of Mib and DHT, as well as MPA, NET-A and LNG, resulted in increased proliferation of the MCF-7 BUS and T47D cells. Taken together, the results of this study highlight not only the important roles of the AR and ERβ in breast cancer, but also that the role of progestins in breast cancer is not straightforward. Considering the complex and intertwined nature of steroid receptor signaling pathways in breast cancer, and the fact that some progestins activate multiple steroid receptors, it is important to unravel the mechanisms whereby progestins elicit their effects in breast cancer. Understanding these mechanisms may facilitate the design of new drugs for the treatment and prevention of AR- and ER-positive breast cancer.

Table 4.1. Summary of the effects of androgens and progestogens on AR, ERβ and ERα expression.

			Androgens		Androgenic progestins			Anti-androgenic progestogens		
			Mib	DHT	MPA	NET-A	LNG	P₄	NES	NOMAC
MDA-MB-453 cell line	AR	Protein expression	↑	↑	-	-	-	-	-	-
		Via AR	yes	yes	-	-	-	-	-	-
	ERβ	mRNA expression	↑	↑	↑	↑	↑	↓	-	-
		Via AR	yes	yes	yes	yes	yes	no	-	-
MCF-7 BUS cell line	AR	Protein expression	↑	↑	-	-	-	-	-	-
		Via AR	yes	yes	-	-	-	-	-	-
	ERβ	mRNA expression	↑	↑	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
		Via AR	?	?	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	ERα	mRNA expression	↓	↓	-	↓	-	↓	-	-
		Via AR	yes	yes	-	no	-	yes	-	-
		Protein expression	-	-	-	↓	-	-	-	-
		Via AR	-	-	-	no	-	-	-	-

Upregulation (↑), downregulation (↓), no effect (−), not determined (N.D.), unanswered (?).

Table 4.2. Summary of the effects of androgens and progestogens on breast cancer cell proliferation.

			Androgens		Androgenic progestins			Anti-androgenic progestins	
			Mib	**DHT**	**MPA**	**NET-A**	**LNG**	**P₄**	**NES**
MCF-7 BUS cell line	**Steroid receptor required:**	Proliferation	↑	↑	↑	↑	↑	↑	↑
		AR	yes	yes	yes	yes	yes	no	no
		ER	?	?	?	?	?	?	?
		PR	?	?	?	?	?	?	?
		GR	?	?	?	?	?	?	?
T47D cell line	**Steroid receptor required:**	Proliferation	↑	↑	↑	↑	↑	↑	↑
		AR	yes	yes	yes	yes	yes	no	no
		ER	no	no	no	yes	yes	no	no
		PR	yes	no	yes	yes	yes	yes	yes
		GR	?	?	?	?	?	?	?

Upregulation (↑), downregulation (↓), no effect (−), unanswered (?).

4.10   Future work

A major limitation of the current study is the fact that we were unable to reproduce our results showing that Mib and DHT increased ERβ mRNA expression in the MCF-7 BUS and T47D breast cancer cell lines. As it has been suggested that experiments in adherent cancer cell lines should be performed within the first ten passages of a new freezer stock (Masters, 2000), future experiments should be performed within this timeline. A further limitation of the study is the fact that results from the MTT cell viability assay in the MDA-MB-453 cell line are from a single experiment. To validate these findings, at least two more biological repeats of this experiment are required. In addition, an *in vitro* mammalian two-hybrid assay was used to assess the AR N/C interaction, which does not evaluate the full-length human AR. Thus, quantitative fluorescence resonance energy transfer (FRET) assays can be used to investigate the AR N/C interaction via the full-length human AR in living cells. This can be achieved by tagging the NTD and COOH-terminal of the AR with the FRET couple cyano fluorescent protein (CFP) and yellow fluorescent protein (YFP). The fluorescence signal upon excitation of CFP or YFP can then be measured with a fluorescence microscope as previously described (Schaufele *et al.*, 2005).

Rizza *et al.* (2014) showed that the androgen-activated AR increased ERβ expression by binding to an ARE in the promoter of the human ERβ gene. Electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays can be performed to evaluate whether the progestin-bound AR also occupies this ARE *cis*-element. EMSA is an *in vitro* method used to detect whether a protein binds to a given DNA sequence, while the ChIP assay is used to evaluate the interaction between the protein and the DNA in the cell (Smith and Humphries, 2009; Nguyen-Duc *et al.*, 2012). We showed that the Mib- and DHT-activated AR decreased ERα mRNA expression. We hypothesized that this downregulation occurs via the ERα tethering to DNA-bound transcription factors like AP-1 (McDonnell and Norris, 2002). A combination of siRNA technology and ChIP assays can be used to investigate whether the Mib- and DHT-activated AR and AP-1 are co-recruited to the AP-1 *cis*-element in the ERα gene promoter (Carroll *et al.*, 2006). Progestins are often administered to women in combination with an estrogen (Greendale *et al.*, 1999; Hickey *et al.*, 2012). Thus, future studies should also investigate the effects of E_2 and progestin combinations on ERα and ERβ expression, as well as on breast cancer cell proliferation. Given that the role of the ER and PR in MCF-7 BUS cells, and GR in either MCF-7 BUS or T47D cell lines (Figure 3.5), could not be assessed using receptor antagonists, silencing the expression of a particular steroid receptor by siRNA technology may be a better strategy in future studies. Finally, to confirm that the findings of this study

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are clinically relevant, it is crucial that future studies evaluate whether the effects of the androgens and progestins on ERα and ERβ expression can be extended into primary human breast tumours.

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Addendum A

Buffers and Solutions

A.1   Bacterial media

Luria Bertani (LB) medium

10 g NaCl

10 g Tryptone

5 g Yeast extract

Adjust to a final volume of 1 L using reverse osmosis (RO) H_2O.

Sterilize by autoclaving and store at room temperature.

LB Agar plates

LB medium

15 g/L Bacterial agar

Ampicillin (final concentration 50 μg/mL)

A.2   Cell lysis

10X Tris-phosphate-EDTA (TPE) buffer

108 g Tris (hydroxymethyl) aminomethane

15.5 mL 85% (v/v) Phosphoric acid

40 mL 0.5 M EDTA (pH 8.0)

Adjust to a final volume of 1 L using RO H_2O.

Sterilize by autoclaving and store at room temperature.

Passive Lysis Buffer

0.5 mL Triton X-100

25 mL Glycerol

7 mL 1X TPE buffer

720 μL 0.5 M EDTA

Adjust to a final volume of 250 mL using RO H_2O and store at 4°C.

A.3   SDS-PAGE and western blotting

10% (w/v) SDS

Dissolve 10 g SDS in 100 mL RO H_2O at 68°C.

Store at room temperature.

2X Laemmli buffer

1 mL 1M Tris-HCl (pH 6.8)

5 mL 10% (w/v) SDS

2 mL Glycerol

500 μL β-mercaptoethanol

0.01 g Bromophenol blue

Adjust to a final volume of 25 mL with RO H_2O and store at -20°C.

10X SDS-PAGE Running Buffer

20 g SDS

60.6 g Tris

288.2 g Glycine

Adjust to a final volume of 2 L using RO H_2O and store at room temperature.

1X Transfer Buffer

6.06 g Tris

28.83 g Glycine

200 mL Methanol

Adjust to a final volume of 2 L using RO H_2O and store at 4°C.

10X Tris buffered saline (TBS) (pH 7.5)

Dissolve 60.5 g Tris and 87.6 g NaCl in 800 mL RO H_2O.

Adjust the pH to 7.5

Adjust to a final volume of 1 L using RO H_2O and store at 4°C.

TBS-Tween (TBST)

100 mL 10X TBS buffer

1 mL Tween 20

Adjust to a final volume of 1 L using RO H_2O and store at 4°C.

A.4   Agarose gel electrophoresis

50X Tris-acetate-EDTA (TAE) buffer

Dissolve 242.2 g Tris in 700 mL RO H_2O.

Add 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.2).

Adjust to a final volume of 1 L using RO H_2O and store at room temperature.

10X Morpholinopropanesulfonic acid (MOPS) running buffer

Dissolve 83.71 g MOPS in 800 mL DEPC-treated H_2O.

Add 33.4 mL 3 M NaOAc and 20 mL 0.5 M EDTA RNase-free stock solutions.

Adjust pH to 7.0

Adjust to a final volume of 1 L using DEPC-treated H_2O.

Sterilize by autoclaving and store in a dark place at 4°C.

2X Formaldehyde RNA loading buffer

0.071 g Bromophenol blue

1 mL Glycerol

1.5 mL 10X MOPS

2.6 mL Formaldehyde

7.3 mL Formamide

Adjust to a final volume of 15 mL using DEPC-treated H_2O.

Prepare 1 mL aliquots and store at -20°C.

Add 2.5 μL ethidium bromide to each 1 mL aliquot before use.

A.5   Cell viability assays

MTT Solution (5 mg/mL)

Dissolve 5 mg MTT in 1 mL sterile 1X PBS.

Filter sterilize.

A.6   Charcoal-stripping of FCS

Lipophilic materials such as growth factors, hormones and cytokines, which may affect hormone-induced responses and subsequently impact experimental results, are removed from FCS using dextran-treated charcoal (Lau and Chang, 2014).

Charcoal-stripping Buffer

250 mL 1 M Sucrose

1.5 mL 1 M Magnesium chloride hexahydrate

10 mL 1 M HEPES

2.5 g Norit-A® (activated charcoal)

0.025 g Dextran

Adjust to final volume of 1 L using Milli-Q® H_2O, and stir overnight at 4°C.
Store at 4°C.

1. Activated dextran-treated charcoal was collected from 500 mL charcoal-stripping buffer by centrifugation at 500 x g for 30 minutes at 4°C.
2. The supernatant was discarded and the charcoal pellet resuspended in 50 mL FCS, which was stirred overnight at 4°C.
3. The next day, the charcoal was removed from the FCS by centrifugation at 500 x g for 30 minutes at 4°C.
4. Steps 1-3 were repeated using the same FCS sample.
5. Excess charcoal was removed from the stripped FCS by filter-sterilization.

A.7   Heat-inactivation of FCS

Heat-inactivation of FCS leads to the inactivation of the complement system as well as the destruction of heat labile growth factors, vitamins, amino acids, and hormones that may be present (Ayache *et al.*, 2006). To heat-inactivate FCS, the serum was thawed and subsequently heated to 56°C for 30 minutes.

Addendum B

Additional Data

B.1   All cell lines used in this study were mycoplasma negative.
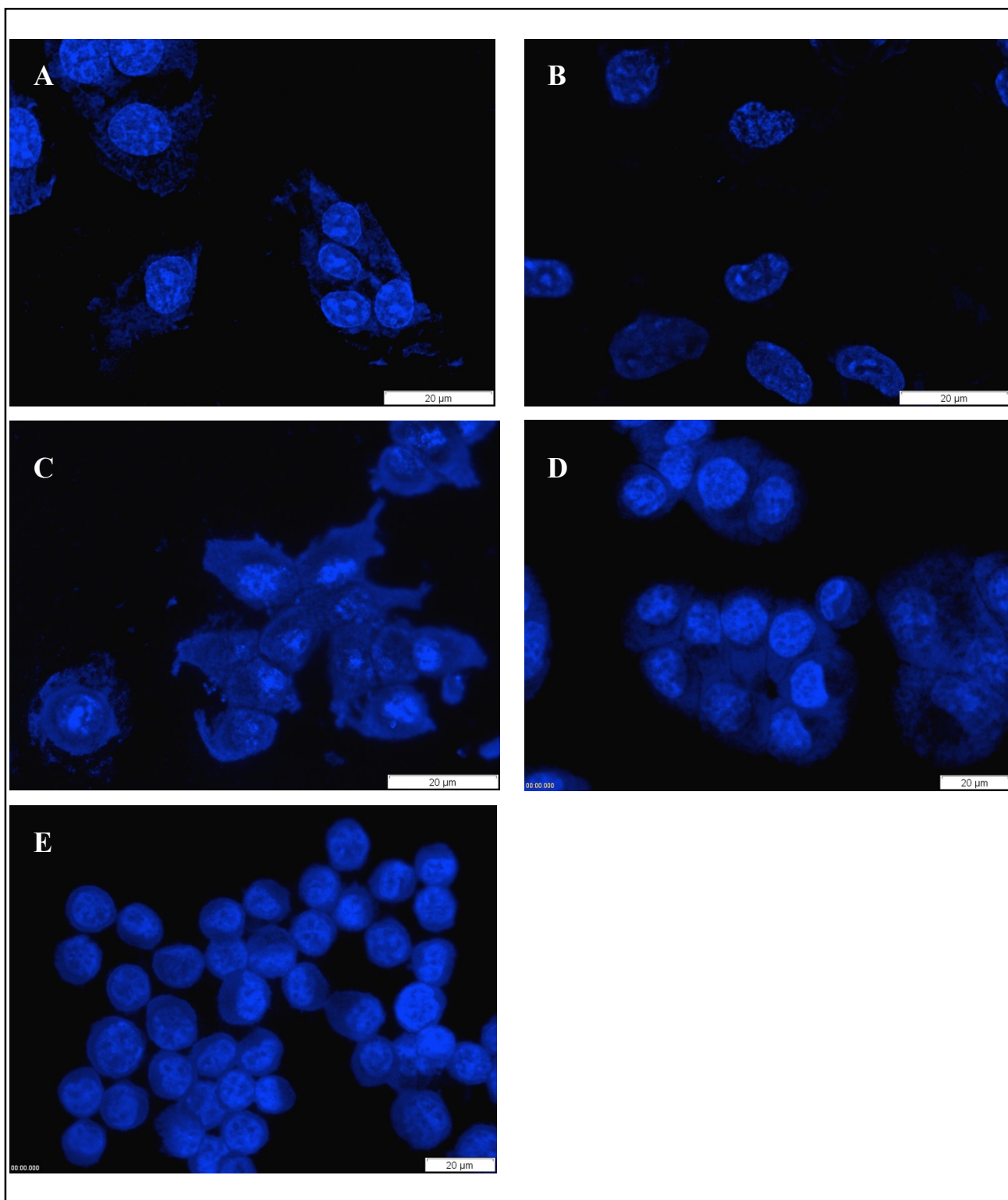
Figure B.1. Mycoplasma negative (A) COS-1, (B) HEK-293, (C) MCF-7 BUS, (D) T47D and (E) MDA-MB-453 cells. Cells were stained with the DNA Hoechst 33258 dye (Sigma-Aldrich, South Africa). Only DNA-containing nuclei are stained with the Hoechst dye. The fluorescence was visualized and the images captured using the Olympus IX81 inverted fluorescence microscope (Life Science Solutions, South Africa).

B.2   RU486 antagonizes the PR, but not GR, in the T47D breast cancer cells, but does not elicit antagonist activity in the MCF-7 BUS cell line.
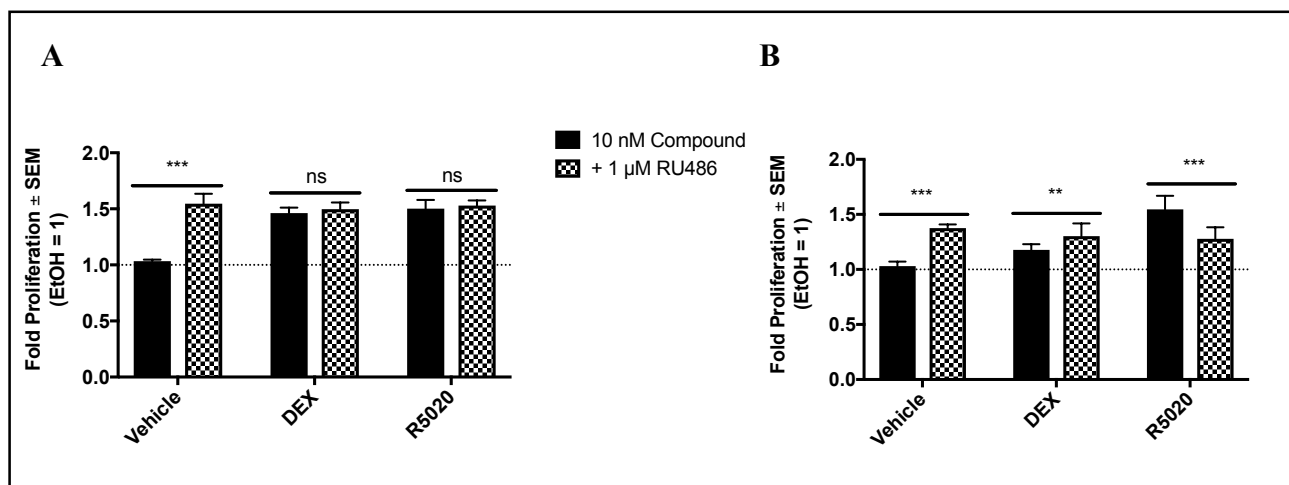
Figure B.2. RU486 increases proliferation of both MCF-7 BUS and T47D cells, and acts as a PR, but not GR antagonist in the T47D cells. The human (A) MCF-7 BUS and (B) T47D breast cancer cells were treated for 72 hours with either 0.2% (v/v) EtOH (vehicle), 10 nM of the GR-specific agonist, Dex, or the PR-specific agonist, R5020, in the absence and presence of 1 μM RU486. The medium was aspirated and cells were re-treated for an additional 44 hours. Cell proliferation was measured using the MTT cell viability assay. Results are represented as fold proliferation relative to the vehicle control. The results shown are the averages (± SEM) of at least three independent experiments with each condition performed in triplicate. Statistical analysis was performed using two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

B.3   ICI significantly decreases the viability of the MCF-7 BUS breast cancer cell line, but not the T47D cell line.
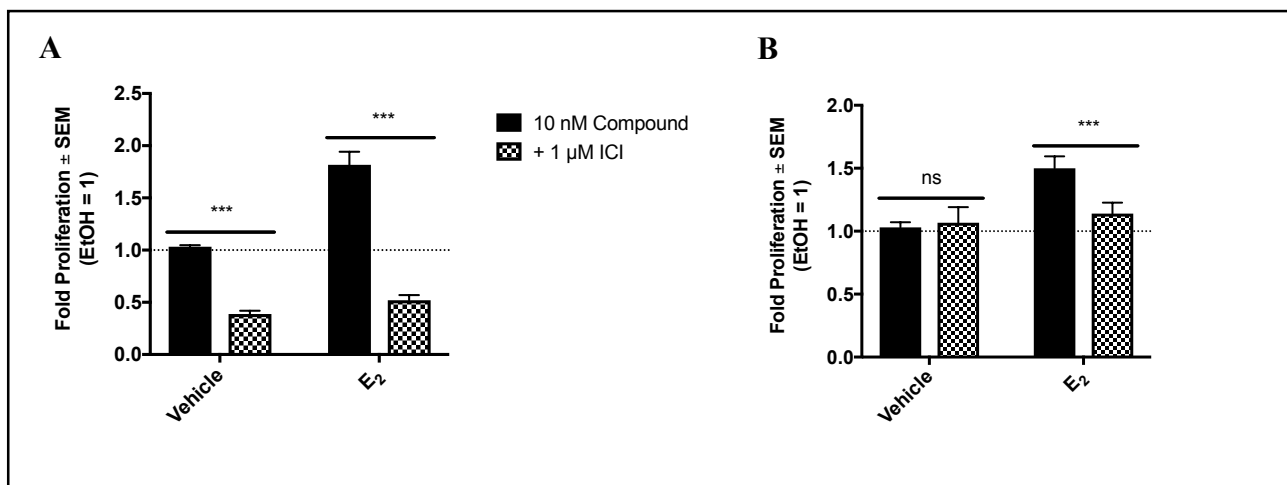
Figure B.3. ICI significantly decreases the viability of the MCF-7 BUS cells, but not the T47D cells. The human (A) MCF-7 BUS and (B) T47D breast cancer cells were treated for 72 hours with either 0.2% (v/v) EtOH (vehicle) or 10 nM of the ER-agonist, E_2, in the absence and presence of 1 μM of the ER antagonist, ICI. The medium was aspirated and cells were re-treated for an additional 44 hours. Cell proliferation was measured using the MTT cell viability assay. Results are represented as fold proliferation relative to the vehicle control. The results shown are the averages (± SEM) of at least three independent experiments with each condition performed in triplicate. Statistical analysis was performed using two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

B.4   The commercial ERβ antibody from Abcam (EPR20743) is not specific for ERβ.
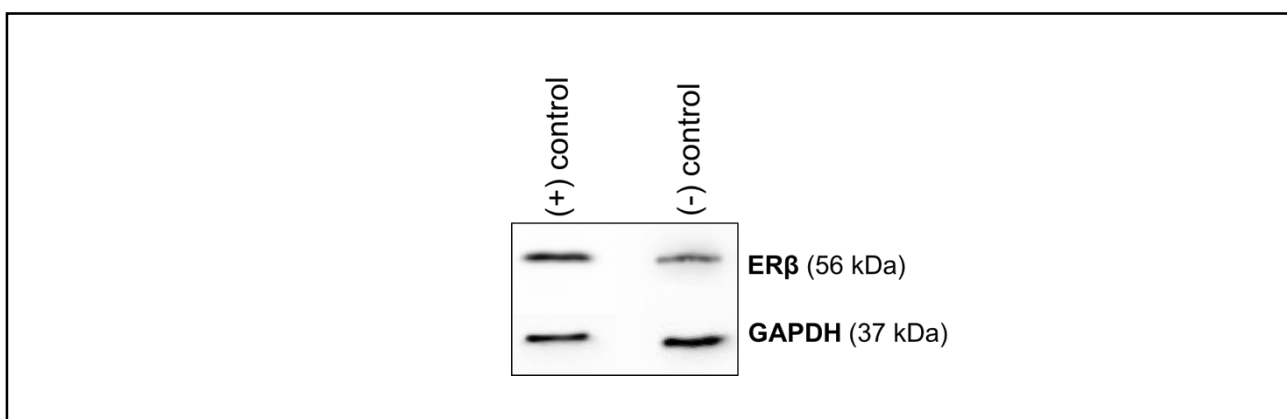
Figure B.4. Western blot analysis indicating non-specificity of the commercial ERβ antibody (EPR20743). Protein lysates were analyzed by western blotting using primary antibodies specific for ERβ and GAPDH (loading control). HEK-293 cells transiently transfected with an expression vector for ERβ was used as a positive control, while untransfected COS-1 cells were used as a negative control. A representative western blot of at least two independent experiments is shown.

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B.5   ERβ mimics the proliferative effects of ERα in the MDA-MB-453 breast cancer cells, while E_2-induced proliferation is decreased when ERα and ERβ are co-expressed.
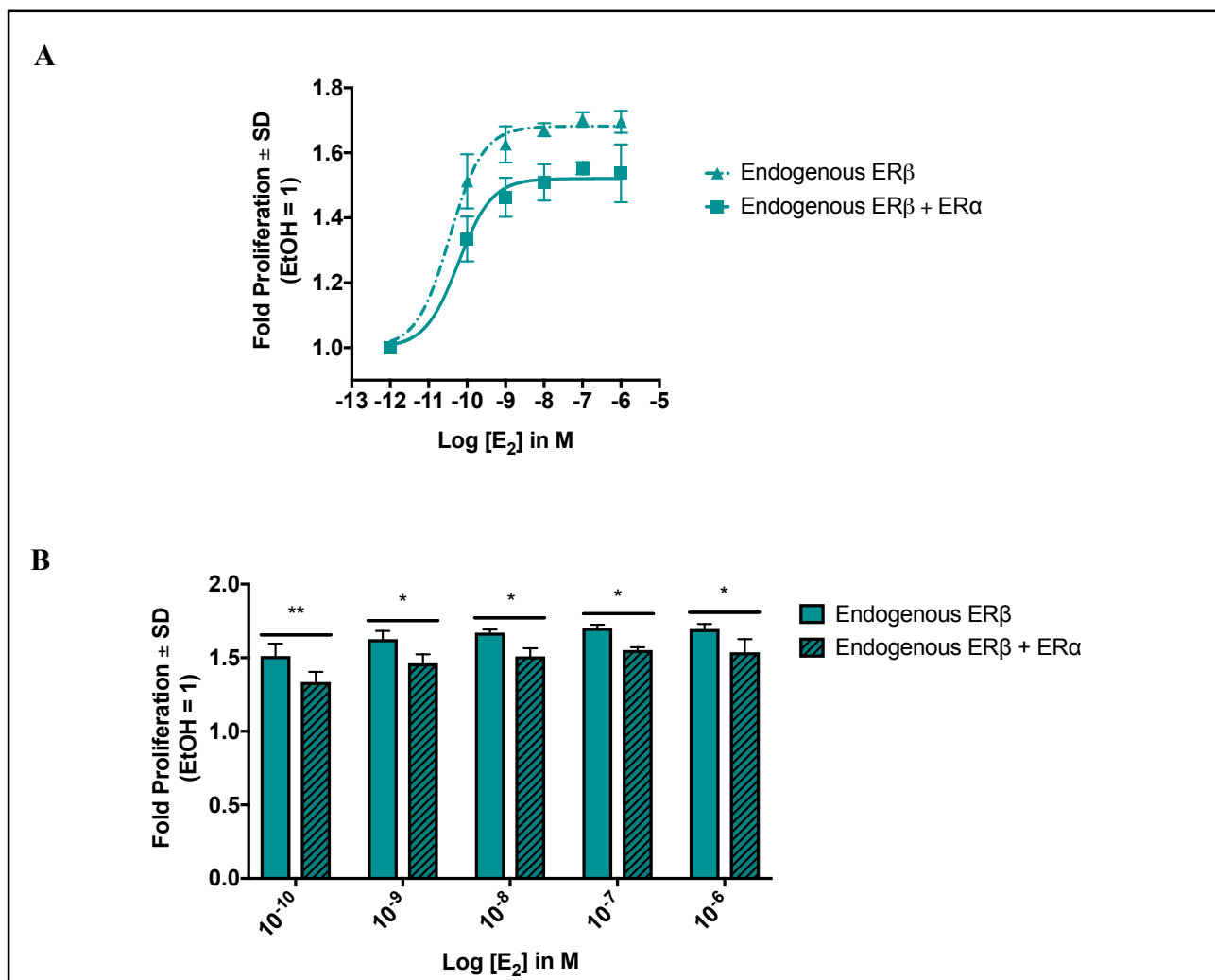

Figure B.5. ERβ mimics the proliferative effects of ERα in the absence of ERα expression, while E_2-induced proliferation is decreased when ERα and ERβ are co-expressed. The human MDA-MB-453 breast cancer cells were either left untransfected or transiently transfected with an expression vector for human ERα. All cells were treated for 72 hours with either 0.1% (v/v) EtOH (vehicle) or increasing concentrations of E_2. The medium was aspirated and cells were re-treated for an additional 44 hours. Cell proliferation was measured using the MTT cell viability assay. Results are represented as fold proliferation relative to the vehicle control. The result shown is the averages (± SD) of a single experiment with each condition performed in triplicate. (B) The data from (A) was re-plotted to directly compare the effects between transfection conditions. Statistical analysis was performed using two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

B.6 Hydroxyflutamide, but not Bicalutamide, antagonizes the proliferative effects of androgens and progestins in T47D breast cancer cells.
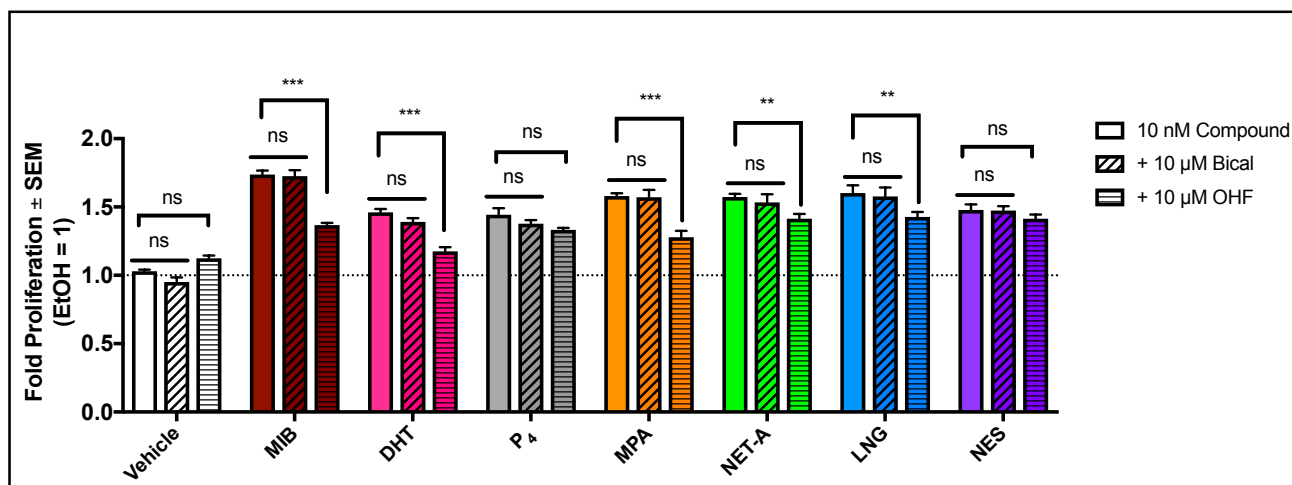

Figure B.6. The AR antagonist, Bicalutamide, does not elicit antagonist activity in the T47D breast cancer cell line. The human T47D breast cancer cells were treated for 72 hours with either 0.2% (v/v) EtOH (vehicle) or 10 nM Mib, DHT, P_4, MPA, NET-A, LNG or NES, in the absence and presence of either 10 µM Bicalutamide (Bical) or 10 µM hydroxyflutamide (OHF). The medium was aspirated and cells were re-treated for an additional 44 hours. Cell proliferation was measured using the MTT cell viability assay. Results are represented as fold proliferation relative to the vehicle control. The results shown are the averages (± SEM) of at least three independent experiments with each condition performed in triplicate. Statistical analysis was performed using two-way ANOVA with Bonferroni's (compares all pairs of columns) as post-test.

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Addendum C

Real-time Quantitative Polymerase Chain Reaction (qPCR)

Optimization

Introduction

Real-time qPCR is a highly sensitive and powerful technique used to quantitatively measure relative mRNA gene expression (Wong and Medrano, 2005). This method involves the amplification of DNA using a fluorescent dye such as SYBR Green, that binds to the minor groove of double stranded DNA (Lekanne Deprez *et al.*, 2002). The amplified DNA can thus be monitored in realtime by measuring the fluorescence (Lekanne Deprez *et al.*, 2002). The detected fluorescence intensity is directly proportional to the amount of amplified DNA (Arya *et al.*, 2005). The qPCR reaction can be divided into four major phases: linear ground phase, early exponential phase, log-linear phase and the plateau phase (Wong and Medrano, 2005; Fraga *et al.*, 2008). The linear ground phase is the phase where the qPCR reaction is initiated (first 10 – 15 cycles) and the fluorescence emitted is below background levels. The amplification cycle number at which the fluorescence exceeds the background fluorescence is known as the quantification cycle (C_q) (Pfaffl, 2001). The log-linear phase is where the amplicon increases exponentially, while the plateau phase occurs when the qPCR reaction components become depleted. In this study, qPCR was used to evaluate the relative mRNA expression of ERα and ERβ following treatment with test compounds.

The first crucial step was the isolation of high quality, intact RNA, as poor quality RNA negatively influences experimental results. Following the isolation of total RNA, the purity thereof was assessed by measuring the optical density (OD), while the RNA integrity was analyzed on a 1% (w/v) denaturing formaldehyde agarose gel. A 260/280 ratio greater than or equal to 1.9 is indicative of pure RNA (Sambrook *et al.*, 1989), while intact total RNA is observed as two clear bands, the 28S and 18S bands, which represent the ribosomal RNA subunits. The 28S band should be approximately twice the intensity of the 18S RNA band. Representative 1% (w/v) denaturing formaldehyde agarose gels are shown in Figure C.1.
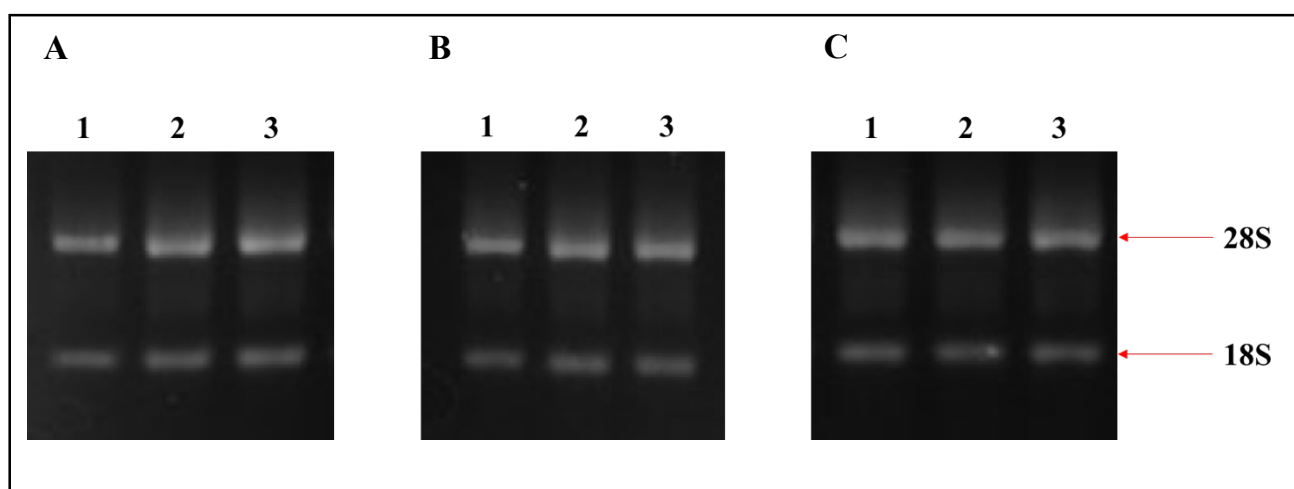

Figure C.1 legend on next page.

Figure C.1. A representative 1% (w/v) denaturing formaldehyde agarose gel showing intact RNA in (A) MCF-7 BUS, (B) T47D and (C) MDA-MB-453 cells. Total RNA was isolated as described in Chapter 2, Section 2.5. One µg RNA was loaded onto the agarose gel, and the RNA was visualized by ethidium bromide staining. Lane 1: Cells treated with EtOH (vehicle control); Lane 2: Cells treated with 10 nM Mib; Lane 3: Cells treated with 10 nM DHT.

The intact total RNA was subsequently reverse transcribed to synthesize cDNA as described in Chapter 2, Section 2.6. Next, real-time qPCR was performed as described in Chapter 2, Section 2.7.

Determination of primer pair efficiency

The efficiency of real-time qPCR is dependent of the amplification efficiency of the primer pair used, which is generally assumed to be two, as the amount of DNA present in a sample theoretically doubles with each PCR cycle (Pfaffl, 2001; Wong and Medrano, 2005). As this may not always be the case, the primer efficiencies of ERα and ERβ were determined before the relative gene expression was calculated. To achieve this, a serial dilution of a single cDNA sample was prepared and each dilution analyzed in triplicate. PCR-grade water was used as negative control to confirm that there was no contamination or primer self-amplification. Standard curves (Figure C.2) were then generated by plotting the C_q value against the log cDNA concentration. The slope of the curve was then determined and used to calculate the primer efficiency (E) using the following mathematical equation (Pfaffl, 2001):

$$E = 10^{(-1/\text{slope})} \qquad \text{(Equation 1)}$$

The primer efficiencies reported for the ERα, ERβ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer pairs (Table C.1) are the averages of two independent experiments. The efficiency for the ERα primer pair was determined in MCF-7 BUS cells, while the ERβ primer efficiencies were investigated in the T47D and MDA-MB-453 cells. Meghan Perkins in the Africander laboratory previously determined the efficiency for the ERβ primer pair in the MCF-7 BUS cells. The efficiency for the GAPDH primer pair was determined in the MDA-MB-453 cells, while Renate Louw-du Toit in the Africander laboratory previously determined efficiencies for this primer pair in the MCF-7 BUS and T47D cells.

Table C.1. Efficiencies for primer pairs used in this study.

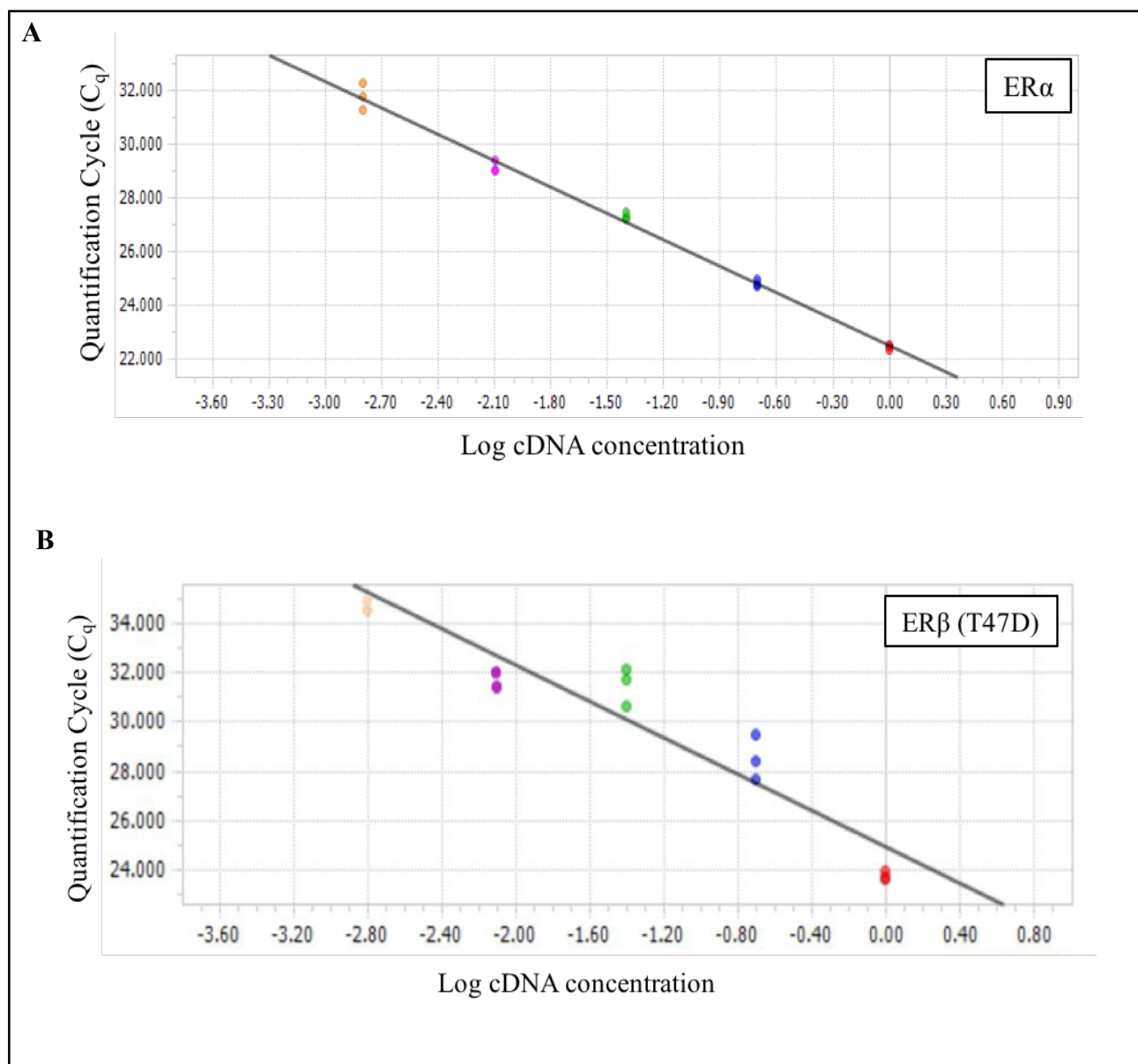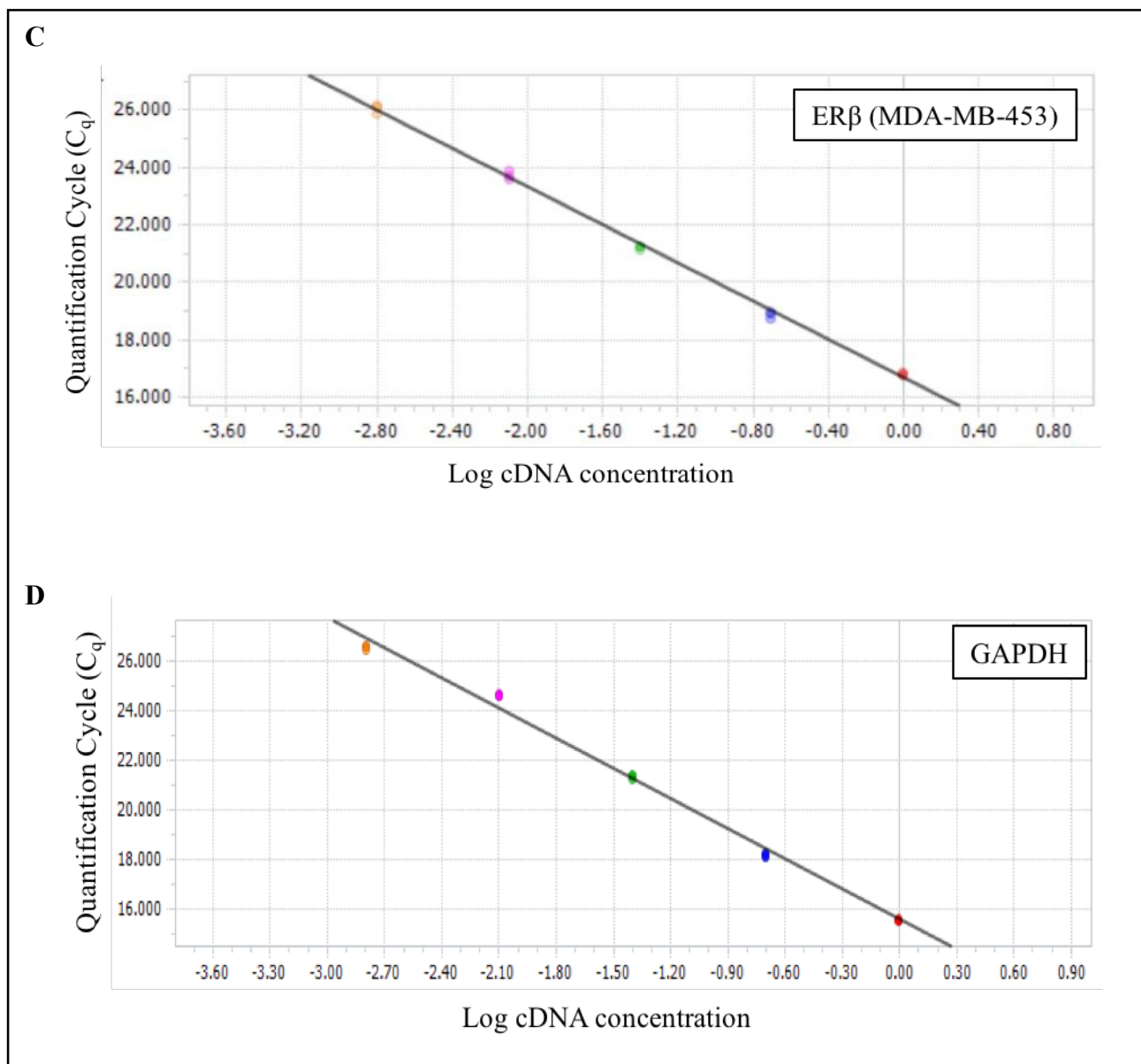| Primer pair	Cell line	Primer efficiency (E)
ERα	MCF-7 BUS	2.02
ERβ	MCF-7 BUS	1.94
	T47D	1.86
	MDA-MB-453	1.99
GAPDH	MCF-7 BUS	1.86
	T47D	1.89
	MDA-MB-453	1.76

Figure C.2 continued on next page.

Figure C.2. Representative standard curves for the (A) ERα, (B and C) ERβ and (D) GAPDH primer pairs. Standard curves were generated by plotting the C_q value against the log concentration of cDNA. The slope determined from these standard curves were used to calculate the efficiency of the primer pairs used in the (A) MCF-7 BUS, (B) T47D as well as the (C and D) MDA-MB-453 cell lines.

Melting curve analysis

In real-time qPCR, each PCR product has a unique melting temperature, which is dependent on the nucleotide content and size of the desired amplicon (Ririe *et al.*, 1997). Analysis of the melting peak of a sample confirms the presence of a single PCR product. The melting curve analysis was performed by varying the temperature between 50°C and 95°C, gradually increasing the temperature by 2.2°C, and continuously measuring the fluorescence after each temperature-increase step. Using the LightCycler® 96 software, a melting curve was plotted with the negative derivative of fluorescence

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over temperature (-dF/dT) on the y-axis and temperature (°C) on the x-axis (Figure C.3). The presence of more than one peak on the melting curve is indicative of primer-dimers or other non-specific PCR products (Ririe *et al.*, 1997; Fraga *et al.*, 2008).
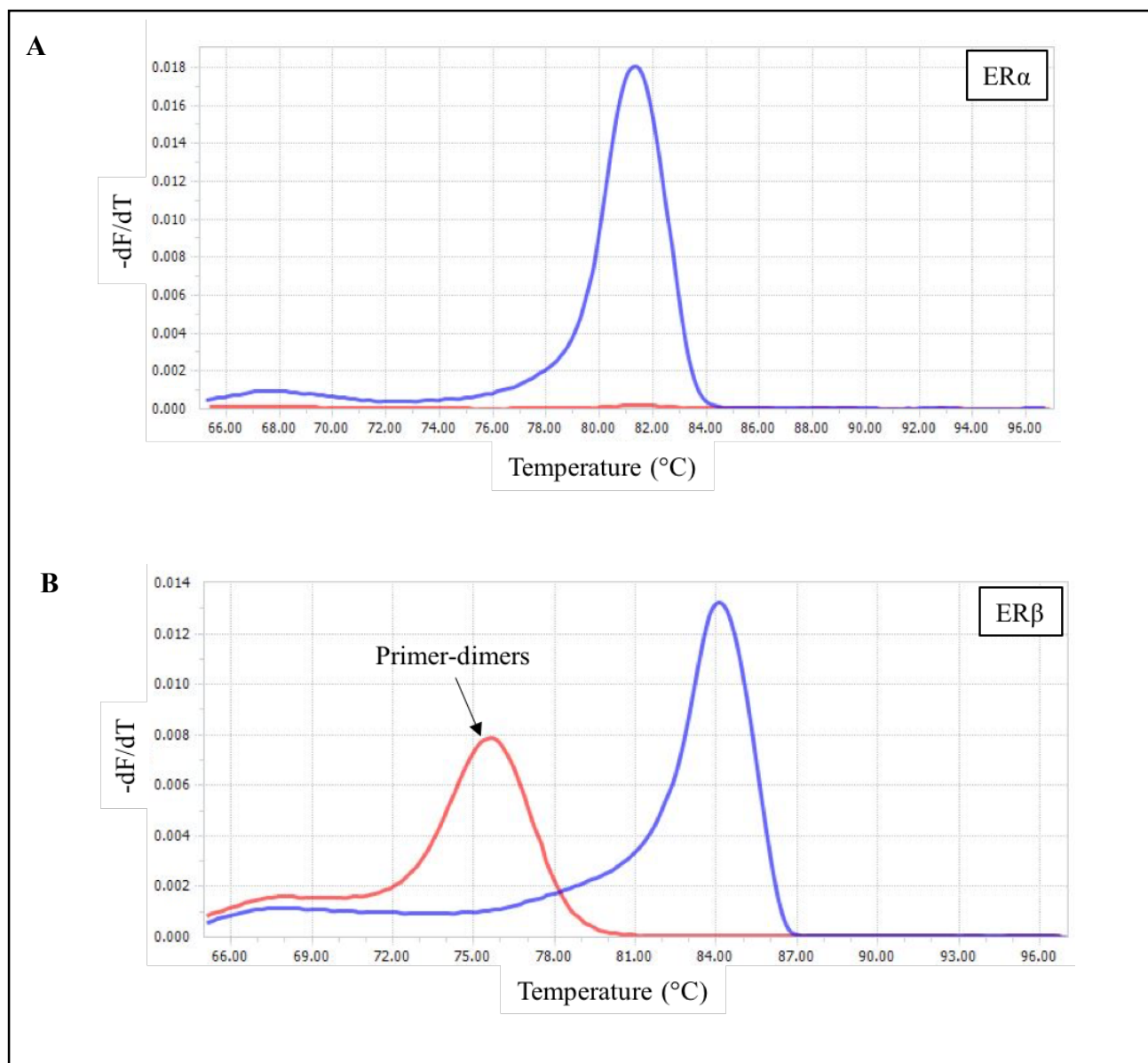

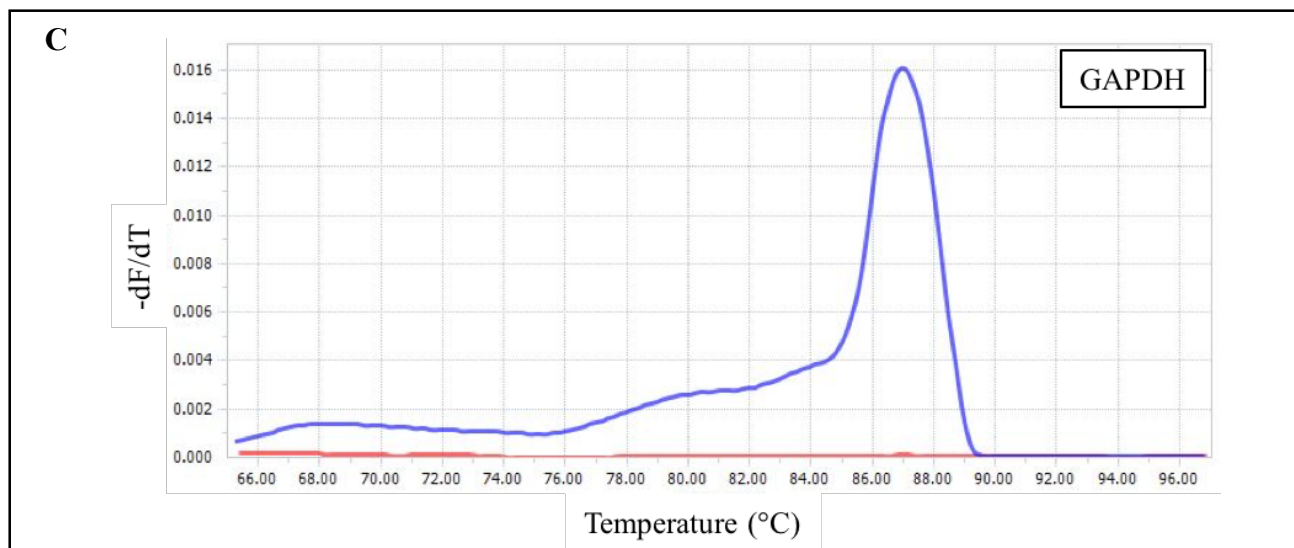**Figure C.3 continued on next page.**

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Figure C.3. Melting curve analysis for (A) ERα, (B) ERβ and (C) GAPDH. Melting curves were generated using the LightCycler® 96 software. (A) ERα gene expression was investigated in MCF-7 BUS cells, while (B) ERβ and (C) GAPDH gene expression was investigated in MDA-MB-453 cells. Samples treated with EtOH (vehicle control) are indicated in blue, while the red curves indicate the negative controls containing no template.

Single product amplification was also confirmed by performing agarose gel electrophoresis. Representative agarose gels are shown in Figure C.4.
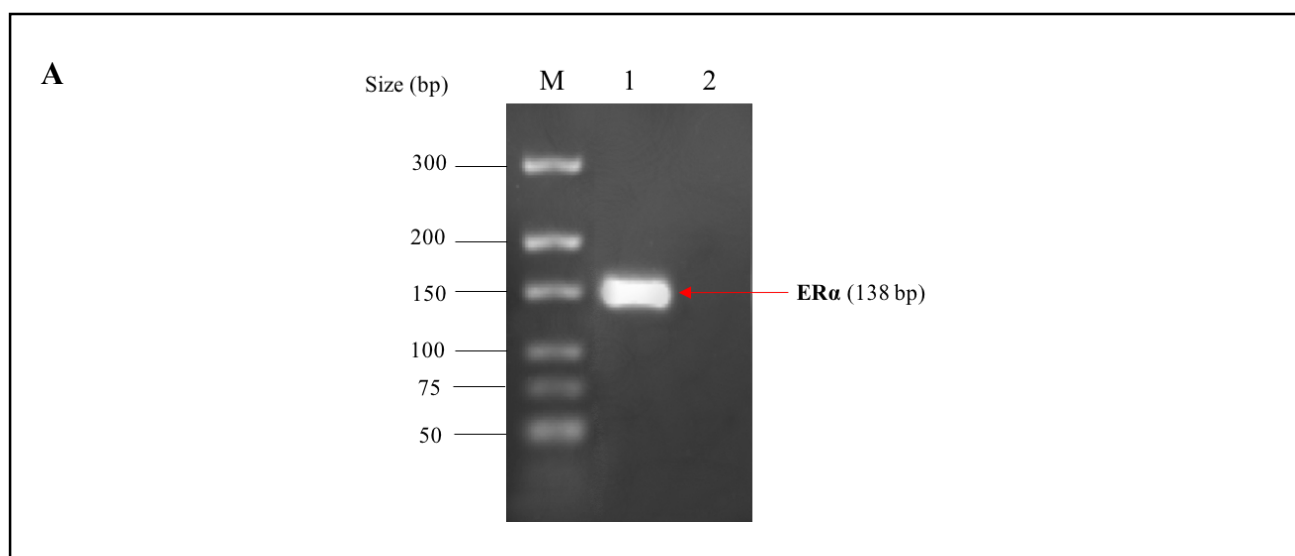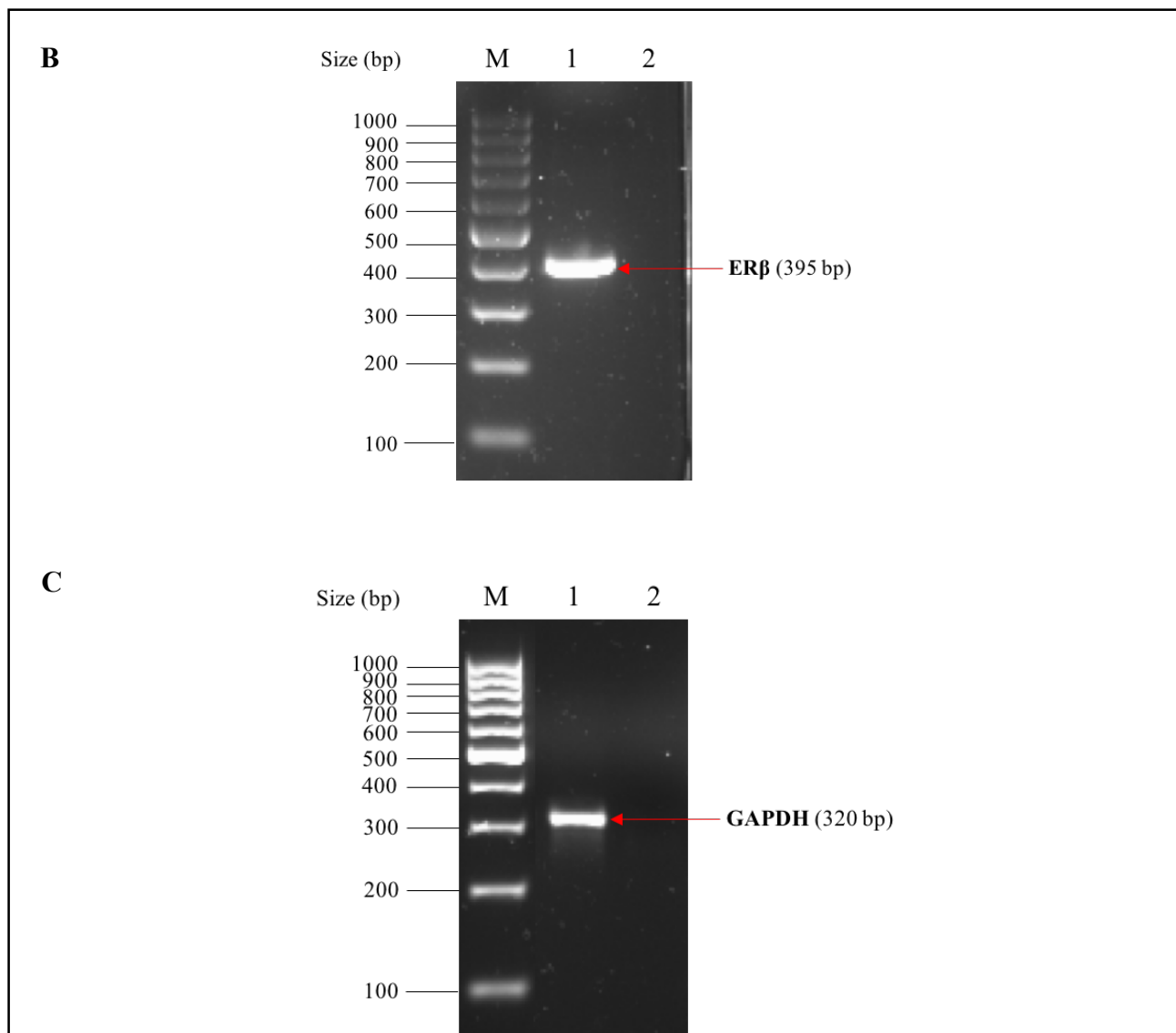
Figure C.4. Representative agarose gels indicating the real-time qPCR end products of (A) ERα, (B) ERβ and (C) GAPDH. PCR end products were subjected to agarose gel electrophoresis (2% (w/v)) and visualized using the Nancy-520 (Sigma-Aldrich, South Africa) nucleic acid stain. (A) M: Ultra-Low Range DNA Ladder (Thermo Fisher Scientific, South Africa); (B and C) M: 100 bp DNA Ladder (Thermo Fisher Scientific, South Africa); Lane 1: Sample treated with EtOH (vehicle control); Lane 2: No template negative control.

Determination of relative gene expression values

The relative expression levels (R) of the target genes were calculated using the mathematical equation described by Pfaffl (2001), and expressed as the ratio of the target gene expression relative to the expression of the reference gene, GAPDH (Equation 2):

$$R = \frac{(E_{target\ gene})^{\Delta Cq_{target\ gene}\ (control-sample)}}{(E_{reference\ gene})^{\Delta Cq_{reference\ gene}\ (control-sample)}} \qquad \text{(Equation 2)}$$

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The R value is dependent on the efficiency of the primer pair (calculated using Equation 1) and the change in quantification cycle (ΔC_q). The value for ΔC_q was calculated by subtracting the C_q value of the treated sample from the C_q value of the vehicle control sample (EtOH). A R value of one indicates that there is no difference between samples treated with test compounds and samples treated with EtOH. A R value greater than one indicates the upregulation of the target gene, while a value smaller than one indicates inhibition (Pfaffl, 2001).